UNIVERSITY OF ALBERTA

PHOSPHORYLATION OF HERPES SIMPLEX VIRUS TYPE-1 IMMEDIATE-EARLY PROTEIN ICP4 IS REQUIRED FOR ITS RECRUITMENT TO NUCLEAR REPLICATION COMPARTMENTS

By

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ABSTRACT

The nuclear domains in which herpes simplex type-1 (HSV-1) genomes are transcribed and replicated are called replication compartments (RCs). RCs contain seven HSV-1 DNA replication proteins, two HSV-1 transcription regulators (ICP4 and ICP27), HSV-1 genomes, and selected cellular proteins. Pre-replication compartments (pre-RCs) are smaller domains formed early in infection containing the same viral proteins but not HSV-1 genomes. A subset of pre-RCs are thought to be the precursors of RCs. The immediate-early phosphoprotein ICP4 may be recruited to the pre-RCs or RCs by the ICP4 binding sites in the HSV-1 genomes, or by binding to proteins at the core of these compartments. ICP4 phosphorylation is required for its transcriptional regulatory activation, but not for its DNA binding activity. It is yet unknown whether ICP4 phosphorylation is required for its recruitment into RCs. My hypothesis is that ICP4 phosphorylation is required for its recruitment into RCs. A cyclin-dependent kinase (CDK) inhibitor, roscovitine, inhibits ICP4 phosphorylation. To test my hypothesis, I thus analyzed the effects of roscovitine on ICP4 localization. As expected, ICP4 was expressed to only very low levels in the presence of roscovitine. Moreover, ICP4 was nuclear diffuse in approximately 80% of ICP4 expressing cells. Inhibition of ICP4 recruitment to RCs could have resulted from the low levels of ICP4 expressed in the presence of roscovitine. I thus analyzed ICP4 expression and localization in single cells. High levels of ICP4 directly correlated with recruitment into RCs in the absence of roscovitine, but not in its presence. Furthermore, overexpressed ICP4 was not phosphorylated and did not localize to RCs in the presence of roscovitine. Roscovitine did not inhibit ICP4 binding to its cognate DNA sites. I further tested the effects of

phosphorylation on ICP4 localization to preformed RCs by adding roscovitine when RCs were already formed in approximately 50% of cells (5 hours after infection). Further ICP4 phosphorylation and recruitment to RCs was inhibited for 2h by roscovitine, but not by a concentration of PAA that inhibited DNA replication to a similar extent. Therefore, the effects of roscovitine on ICP4 recruitment to RCs are not exclusively a consequence of its effects on ICP4 expression levels, HSV-1 DNA replication, or formation of RCs. Thus, ICP4 recruitment to RCs requires roscovitine-sensitive phosphorylation, but it is not an exclusive consequence of ICP4 binding to its cognate sites.

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ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CAK	CDK activating kinase
cAMP	Cyclic adenosine monophosphate
cdc	Cell division cycle
CDK	Cyclin-dependent kinase
CHX	Cyclohexamide
CK	Casein kinase
CLK	CDK-like kinase
CTD	C-terminal domain
Da	Dalton
DAS	Downstream activating sequence
DBP	DNA binding protein
DMEM	Dulbecco's modified minimum eagle's medium
DMSO	Dimethyl sulfoxide
DNA-PK	DNA-dependent protein kinase
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
E	Early
E2F	Elongation factor 2
EAP	EBER-associated protein
EBV	Epstein-Barr virus
Flavo	Flavopiridol
G	Gap
GTF	General transcription factor
GST	Glutathione-S-transferase
h	hours
HCF	Host cell factor

HCMV	Human cytomegalovirus
HDAC	Histone deacetylase
HEL	Human embryonic lung
HFF	Human foreskin fibroblast
HIV	Human immunodeficiency virus
hpi	hours post-infection
HMG1	High mobility group 1
HSV-1	Herpes simplex virus type-1
HSV-2	Herpes simplex virus type-2
IC	Inhibitory concentration
ICP	Infected cell protein
IE	Immediate-early
Ig	Immunoglobulin
INR	Initiator element
KDa	Kilodalton
L	Late
LBR	Lamin B receptor
M	Mitosis
MAT1	Ménage à trois 1
MEF	Mouse embryonic fibroblast
μLC-MS/MS	Microcapillary high pressure liquid chromatography tandem mass spectrometry
MOI	Multiplicity of infection
mRNA	messenger RNA
MRP1	Multidrug resistance protein 1
MS/MS	Tandem mass spectrometry
ND10	Nuclear dot 10
NLS	Nuclear localization signal
PAA	Phosphonoacetic acid
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen

PIC	Pre-initiation complex
PCI	Pharmacological CDK inhibitor
PFU	Plaque forming unit
PKA	Protein kinase A
PKC	Protein kinase C
PML	Promyelocytic leukemia
POD	Promyelocytic oncogenic domain
pRb	Retinoblastoma protein
pre-RC	Pre-replication compartment
pre-RF	Pre-replication foci
PRV	Pseudorabies virus
RC	Replication compartment
RNAPII	RNA polymerase II
Rosco	Roscovitine
RPA	Replication protein A
S	Synthesis
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering RNA
Sp	Promoter specific protein
ssDNA	single-stranded DNA
TAF	TBP-associated factor
Tat	Transactivator of transcription
TBP	TATA-binding protein
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween
TFII	Transcription factor II
ts	Temperature sensitive
UL	Unique long
US	Unique short
vhs	virion host shut off
VP	Virion protein

VZV Varicella-zoster virus

CHAPTER 1: INTRODUCTION

1.1. Herpes simplex virus type-1 infection

1.1.1. Regulation of herpes simplex virus type-1 gene expression

Herpes simplex virus type-1 (HSV-1) is a large, enveloped, double-stranded DNA virus that replicates in the nucleus. HSV-1 proceeds through two life cycles, lytic and latent. HSV-1 encodes approximately 100 proteins, which are classified according to kinetics of expression as immediate-early (IE), early (E), or late (L) (Honess and Roizman, 1973; Honess and Roizman, 1974; Kozak and Roizman, 1974; Honess and Roizman, 1975; Clements et al., 1977; Jones and Roizman, 1979; Watson et al., 1979).

Expression of HSV-1 proteins is regulated primarily at the transcriptional level, although important RNA processing, translational and post-translational control also occur (Honess and Roizman, 1974; Silverstein and Engelhardt, 1979; Johnson and Spear, 1984; Harris-Hamilton and Bachenheimer, 1985; Wagner, 1985; Godowski and Knipe, 1986; Weinheimer and McKnight, 1987; Su and Knipe, 1989). Transcription of HSV-1 genes is regulated by cis-acting signals within the HSV-1 genome, which are recognized by trans-acting factors encoded by viral and cellular proteins (Zipser et al., 1981; Mackem and Roizman, 1982a; Cordingley et al., 1983; Smiley et al., 1983; Whitton et al., 1983; Campbell et al., 1984; Everett, 1984b; Everett, 1984a; Preston et al., 1984; Dynan and Tjian, 1985; Jones and Tjian, 1985; Jones et al., 1985; Coen et al., 1986).

The products of the immediate-early (IE) genes (ICP0, ICP4, ICP22, ICP27, and ICP47) are expressed immediately after infection. Transcription of the IE genes is activated by a tegument protein, VP16, and two cellular proteins, HCF and Oct-1 (Post et

al., 1981; Batterson and Roizman, 1983; Campbell et al., 1984; O'Hare and Goding, 1988; Preston et al., 1988; Stern et al., 1989; Katan et al., 1990; Xiao and Capone, 1990; Wilson et al., 1993). The promoters of the IE genes contain a minimal transcriptional regulatory unit that governs the very low levels of basal expression (unregulated expression) and highly conserved upstream sequences required for their proper regulation during infection (Mackem and Roizman, 1982b; Mackem and Roizman, 1982a; Kristie and Roizman, 1984; Preston et al., 1984). These upstream sequences are similar to other eukaryotic enhancer elements (Mackem and Roizman, 1982b; Cordingley et al., 1983; Whitton et al., 1983; Preston et al., 1984). They typically contain binding sites for the promoter specific protein 1 (Sp1) (Jones and Tjian, 1985; Jones et al., 1985) and multiple copies of the unique consensus sequence TAATGARAT, which is required for VP16 transactivation (Post et al., 1981; Murchie and McGeoch, 1982; Batterson and Roizman, 1983; Cordingley et al., 1983; Campbell et al., 1984; Lang et al., 1984; Preston et al., 1984).

The majority of IE proteins are critical regulators of HSV-1 gene expression. Two of them activate the transcription of the HSV-1 early (E) genes (Garfinkle and McAuslan, 1974; Honess and Roizman, 1975; Watson and Clements, 1978; Preston, 1979a; Dixon and Schaffer, 1980; Watson and Clements, 1980; Sandri-Goldin et al., 1983; Gelman and Silverstein, 1985; O'Hare and Hayward, 1985a; Quinlan and Knipe, 1985). They also negatively regulate their own expression, mostly at the level of transcription (Preston, 1979b; Preston, 1979a; Dixon and Schaffer, 1980; Read and Frenkel, 1983; DeLuca et al., 1984; DeLuca and Schaffer, 1985; Godowski and Knipe, 1986; Su and Knipe, 1989). IE proteins also possess other important activities. For

example, ICP0 has enzymatic activity as an ubiquitin ligase. ICP0 also induces disruption of nuclear structures called nuclear dots 10 (ND10s) (Maul et al., 1993; Everett and Maul, 1994; Maul and Everett, 1994; Muller and Dejean, 1999), and of centromeres (Everett et al., 1999a; Everett et al., 1999b).

The products of the E genes are mostly involved in HSV-1 DNA replication and metabolism, including the viral DNA polymerase, the ssDNA binding protein ICP8 (Honess and Roizman, 1973), the alkaline exonuclease, the thymidine kinase, and the ribonucleotide reductase (Kit and Dubbs, 1963; Weissbach et al., 1973; Purifoy and Powell, 1976; Powell and Purifoy, 1977; Banks et al., 1983; Dutia, 1983), among others.

The late (L) gene products are mostly structural proteins and are expressed following HSV-1 DNA replication (Sacks et al., 1985; Rice and Knipe, 1988; Sekulovich et al., 1988; McCarthy et al., 1989; Su and Knipe, 1989; McMahan and Schaffer, 1990; Rice and Knipe, 1990). Consequently, L gene transcription is inhibited by HSV-1 DNA polymerase inhibitors such as phosphonoacetic acid (PAA) (Conley et al., 1981; Harris-Hamilton and Bachenheimer, 1985). As infection progresses, expression of IE and E genes decreases and that of L genes increases. The decrease in IE and E gene transcription is thought to be mediated partly by the phosphorylation of the transcription factor Sp1 (Kim and DeLuca, 2002).

1.1.2. HSV-1 mediated "shut" off of host gene expression

Most cellular genes are "shut-off" during HSV-1 infection (Bayliss et al., 1975), in that they are no longer transcribed and their mRNAs are destabilized. This last effect is mediated by the virion host shut-off (*vhs*) protein (Read and Frenkel, 1983; Fenwick

and McMenamin, 1984; Schek and Bachenheimer, 1985; Strom and Frenkel, 1987; Jones et al., 1995). Another HSV-1 protein, ICP27, inhibits RNA splicing (Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994) and host cell transcription (Rice, 1994; Rice et al., 1995; Spencer et al., 1997; Long et al., 1999), which thus reduces the mRNA levels of the cellular genes.

1.1.3. HSV-1 modulation of cellular RNA polymerase II (RNAPII)

Since HSV-1 does not encode the proteins necessary for transcription, it requires cellular transcription complexes. HSV-1 transcription employs specifically cellular RNA polymerase II (RNAPII) (Costanzo et al., 1977; Godowski and Knipe, 1986; Weinheimer and McKnight, 1987; and reviewed in Smiley, 1991). RNAPII is normally phosphorylated on its C-terminal domain (CTD) by TFIIH, which consists of CDK7/cyclin H/MAT1 (Lu et al., 1992). This CTD contains a consensus repeat of seven amino acids, which in turn contains two phosphorylation sites, serine-2 and serine-5. Phosphorylation in these residues converts the hypophosphorylated $RNAPII_a$ (which binds to promoter sequences) to the hyperphosphorylated $RNAPII_o$, (which is released from the promoter to elongate transcripts).

CTD phosphorylation is altered to a novel phosphorylation state ($RNAPII_i$) during HSV-1 infection. This modification consists of a rapid loss of phosphorylation in serine-2. This modification may prevent cellular transcription while allowing for efficient transcription of HSV-1 genes (Rice et al., 1995; Fraser and Rice, 2005). The IE proteins are required and sufficient to inhibit the phosphorylation at serine-2 repeats (Fraser and Rice, 2005).

RNAPII$_I$ localizes to the so-called HSV-1 replication compartments (RCs), localization that requires IE and E proteins (Rice, 1994; Spencer et al., 1997) and induction of RNAPII$_I$ is thought to be mediated in part by the IE protein ICP22. However, proper localization of RNAPII$_I$ also requires the viral protein kinases UL13 (Long et al., 1999) and U_S3 (Leopardi et al., 1997). It was recently suggested that ICP22 interacts with cyclin-dependent kinase 9 (CDK9) to induce phosphorylation of the CTD of RNAPII in an U_S3 dependent fashion (Durand et al., 2005). The localization of RNAPII$_I$ to RCs is not required for repression of host transcription, but it may be required for E and L gene transcription (Spencer et al., 1997; Jenkins and Spencer, 2001).

The IE protein ICP27 associates with RNAPII as early as 3 hours post-infection (hpi). This interaction may be important for activating early and late gene expression or for inhibiting host transcription. It has been hypothesized that ICP27 and ICP4 could recruit RNAPII to the RCs (Randall and Dinwoodie, 1986; de Bruyn Kops et al., 1998; Spencer et al., 2000). The E protein ICP8 also associates with RNAPII, possibly reflecting the role of ICP8 in stimulating late gene transcription. Interaction of ICP8 with RNAPII requires ICP27 (Zhou and Knipe, 2002).

1.2. The immediate-early protein ICP4

1.2.1. ICP4 and its primary structure

The nuclear 175 kilodalton (kDa) IE ICP4 protein is required for the regulation of many steps during HSV-1 infection (Courtney and Benyesh-Melnick, 1974; Pereira et al., 1977). ICP4 is required for transactivation of transcription of E and L genes, repression

of IE genes, and for DNA replication (Preston, 1979b; Preston, 1979a; Dixon and Schaffer, 1980; Watson and Clements, 1980; DeLuca et al., 1984).

Many studies have demonstrated that ICP4 contains discrete functional domains, including DNA binding, dimerization, nuclear localization, and transcription activation domains **(Figure 1.1)** (DeLuca and Schaffer, 1988; Paterson and Everett, 1988b; Paterson and Everett, 1988a; Shepard et al., 1989). The domain between amino acid residues 275 to 490 is required for repression of transcription of IE genes and for activation of transcription of E and L genes (Paterson and Everett, 1988b; Paterson and Everett, 1988a). The domains between amino acid residues 143-210 and 840-1100 appear to be important for activation of transcription of E and L genes but not for repression of transcription of IE genes (Paterson and Everett, 1988a; Shepard et al., 1989). The C-terminal domain of ICP4, in particular that between amino acid residues 1252-1254, functions as an enhancer of the N-terminal transactivation domain (Bruce and Wilcox, 2002). The DNA binding domain is located between amino acid residues 263-487 (Shepard et al., 1989; Wu and Wilcox, 1991). The dimerization domain is located within amino acids 309-489 (Shepard et al., 1990; Wu and Wilcox, 1990). These last two domains are contained within the transactivation/repression domain. Amino acid residues 723-732 contain the nuclear localization signal (NLS) (Showalter et al., 1981; DeLuca and Schaffer, 1988; Mullen et al., 1994), and amino acid residues 171-251 contain a serine-rich region that is required for phosphorylation (DeLuca and Schaffer, 1988).

Temperature sensitive (*ts*) mutants, deletions, and nonsense mutations of ICP4, or inhibition of its expression, result in inhibition of transcription of E and L genes and

overaccumulation of IE mRNAs (Honess and Roizman, 1975; Watson and Clements, 1978; Jones and Roizman, 1979; Preston, 1979b; Preston, 1979a; Dixon and Schaffer, 1980; Preston, 1981; DeLuca et al., 1985; Harris-Hamilton and Bachenheimer, 1985; DeLuca and Schaffer, 1988). Therefore, ICP4 has a central role in the HSV-1 replication cycle activating transcription of most other HSV-1 genes while inhibiting that of the IE ones (Preston, 1979b; Dixon and Schaffer, 1980; Watson and Clements, 1980; DeLuca et al., 1984; Everett, 1984b; DeLuca and Schaffer, 1985; O'Hare and Hayward, 1985a; Quinlan and Knipe, 1985; Godowski and Knipe, 1986).

1.2.2. Repression of IE gene expression by ICP4

Each IE gene promoter displays features of typical RNAPII TATA box promoters, plus the upstream TAATGARAT elements. The TAATGARAT elements are recognized by a complex of cellular HCF and Oct-1 with HSV-1 VP16 (reviewed in Smiley, 1991). The IE promoters also contain multiple Sp1 binding sites (Jones and Tjian, 1985), and GC-rich sequences similar to core enhancer sequences (Mackem and Roizman, 1982a; Lang et al., 1984; Preston et al., 1984). Some also contain a high affinity ICP4 binding site located immediately upstream or overlapping the start site.

ICP4 regulates expression of HSV-1 genes either in a positive or negative manner, depending on the target promoter, ICP4 levels, and presence of ICP0 (Everett, 1984b; O'Hare and Hayward, 1985a; Quinlan and Knipe, 1985; Gelman and Silverstein, 1986; Mavromara-Nazos et al., 1986). The consensus sequence of the ICP4 binding sites is ATCGTC (Faber and Wilcox, 1986a), which was originally suggested to be located only in the ICP0, ICP4, and the L glycoprotein gD promoter-regulatory domains, but not

in the promoters of the other three IE genes (Faber and Wilcox, 1986a; Kristie and Roizman, 1986a; Kristie and Roizman, 1986b; Muller, 1987; Faber and Wilcox, 1988; Tedder et al., 1989). A homologous sequence to ATCGTC has since been found in the promoter of ICP27, but not in those of ICP22 or ICP47 (Gelman and Silverstein, 1987b). The consensus sequence was further found to be conserved among herpesviruses, as is its recognition by the ICP4 homologues, varicella-zoster virus (VZV) IE62 and pseudorabies (PRV) IE180. Therefore, there is a common underlying mechanism to autoregulate transcription of an essential IE gene (Wu and Wilcox, 1991). Gelman and Silverstein first suggested that this consensus binding site is required for repression of IE promoters (Gelman and Silverstein, 1987a). This suggestion has since been experimentally supported by others (Michael et al., 1988; Imbalzano et al., 1990; DiDonato et al., 1991; Rivera-Gonzalez et al., 1994).

ICP4 negatively autoregulates its own transcription by binding to the strong cognate site located at the transcription start site (Dixon and Schaffer, 1980; DeLuca and Schaffer, 1985; O'Hare and Hayward, 1985b; O'Hare and Hayward, 1987; Roberts et al., 1988). This binding may require the E protein ICP8 (Godowski and Knipe, 1986). In support of such mechanism, residues 262 to 490 are sufficient for direct DNA binding to the high affinity sites (Wu and Wilcox, 1990), and thus for repression of IE gene transcription (DeLuca et al., 1985; O'Hare and Hayward, 1985b; DeLuca and Schaffer, 1988; Roberts et al., 1988; Gu et al., 1993; Michael and Roizman, 1993; Rivera-Gonzalez et al., 1994). High concentrations of ICP4 are also required for this repression of IE promoters (DeLuca and Schaffer, 1985). ICP4 can also repress the activation of itself by VP16 *in vitro* (O'Hare and Hayward, 1985b; Gu et al., 1995). Furthermore, binding by

ICP4 causes an alteration in the structure of the DNA helix at the TATA box in IE promoters (DiDonato and Muller, 1989).

It was suggested that the distance and the relative stereoaxial alignment of the ICP4 binding site with respect to the TATA box may determine the extent of repression in IE promoters (Leopardi et al., 1995). At later times in infection, however, the presence of an ICP4 binding site at any position enables repression, perhaps through a yet unknown alternate or additive mechanism (Leopardi et al., 1995).

1.2.3. Activation of transcription of E and L genes by ICP4

The promoters of the E genes are relatively simple, comprising TATA boxes (McKnight and Kingsbury, 1982) and other upstream elements (Everett, 1984a; and reviewed in Smiley, 1991). These elements include cis-acting sequences that are bound by basal cellular transcription factors such as Sp1 (Jones et al., 1985; Imbalzano et al., 1991), and a "CCAAT box" domain homology signal sequence (McKnight and Kingsbury, 1982). The L gene promoters are even simpler, containing only one TATA box and minimal sequences surrounding the start site, including the initiator element (INR) (Homa et al., 1988; and reviewed in Smiley, 1991). Strict L gene promoters, such as the UL38 promoter, also contain a downstream activating sequence (DAS) element (Guzowski et al., 1994).

ICP4 activates transcription of E and L genes at or before the initiation step (Harris-Hamilton and Bachenheimer, 1985; Beard et al., 1986; Tedder and Pizer, 1988). ICP4 also regulates transcription of minimal artificial promoters containing only a TATA

box, by binding either 3' or 5' of it (Coen et al., 1986; Mavromara-Nazos et al., 1986; Shapira et al., 1987; DiDonato and Muller, 1989; Imbalzano et al., 1991).

Transcription of cellular genes requires the assembly of RNA polymerase II and the general transcription factors (GTFs) on the promoter to form a pre-initiation complex (PIC). Among the GTFs, TFIID is the major sequence-specific DNA-binding component. The other GTFs participate on the PIC primarily through protein-protein interactions. TFIID is composed of the TATA-box-binding protein (TBP) and multiple TBP-associated factors (TAFs). ICP4 binds the TBP and TFIIB through amino acids 142 to 274 (which are found within the transactivation and phosphorylation domains), and with the help of TAFs also participates in the formation of transcription complexes (Smith et al., 1993; Gu and DeLuca, 1994; Gu et al., 1995). This interaction is mediated by DNA-protein interactions (TBP to TATA box and ICP4 to cognate sites) and protein-protein interactions between these proteins (Smith et al., 1993). ICP4 then recruits TFIID/TBP and TFIIB to the DNA template and enhances their DNA-binding potential by causing a conformational change of the DNA (Smith et al., 1993). The high mobility protein 1 (HMG1) may bend HSV-1 DNA thus changing the promoter conformation. This change in DNA conformation may then allow ICP4 to more efficiently interact with TFIID and thus enhance TFIID binding to the TATA box (Carrozza and DeLuca, 1998).

ICP4 activation of transcription of L genes is mediated by stimulation of the formation of PICs in a TATA-box dependent manner. The facilitation of TFIID binding is a function of the domains contained in amino acid residues 30-274 (which coincides partly with the transactivation and phosphorylation domains). However, ICP4 DNA binding activity is not sufficient for the facilitation of TFIID binding (Grondin and

DeLuca, 2000). The carboxy terminal domain of ICP4 interacts with TFIID via $TAF_{II}250$, which may support ICP4-activated transcription (Carrozza and DeLuca, 1996). For the strict L promoters, it has been suggested that the multi-functional protein DNA-dependent protein kinase (DNA-PK) may interact with RNAPII to recruit it to DAS containing promoters (Petroski and Wagner, 1998). Furthermore, TFIID binds to the DAS sequences via $TAF_{II}70$. In conjunction with DNA-PK mediated recruitment of RNAPII, thus, TFIID initiates transcription of the promoters (Petroski et al., 2001).

No specific ICP4 binding sites responsible for activation of E and L promoters have been identified (Everett, 1984a; Imbalzano et al., 1990; Smiley et al., 1992), although deletion of ICP4 blocks expression of E and L transcripts (DeLuca et al., 1985). Certain deletion or temperature sensitive mutations in approximately the same last 500 amino acids that encompass one of the transactivation domains of ICP4 have no effect on activation of E gene transcription but fail to activate L gene transcription. These results suggest that ICP4 may act differently on E and L promoters (DeLuca et al., 1984; DeLuca and Schaffer, 1988). It is possible that the cellular transcription complexes are different due to the difference in E and L promoter structures. Zabierowski and DeLuca have demonstrated that the general transcription factor TFIIA is required for ICP4 activation of E promoters but not of L ones (Zabierowski and DeLuca, 2004). ICP4 was found to sufficiently stabilize TFIID through the INR sequence in L genes without the activity of TFIIA. Furthermore, TFIIA mRNA was greatly reduced at late times post-infection. They further suggested that the abundance of TFIIA may decline at late times in infection, potentially downregulating ICP4 activation of E promoters. Such

downregulation would still allow for efficient transcription of L genes, while turning down transcription of E genes.

1.3. Post-translational modifications of ICP4

1.3.1. Poly(ADP)-ribosylation and nucleotidylation

ICP4 is poly(ADP)-ribosylated (Preston and Notarianni, 1983; Blaho et al., 1992). Cellular proteins that are poly(ADP)-ribosylated often play important roles in cellular DNA replication and recombination (for a review on nuclear poly(ADP)-ribosylation see Purnell et al., 1980). However, the specific ICP4 functions which may require poly(ADP)-ribosylation are unknown. The extent of poly(ADP)-ribosylation may correlate with ICP4 transcription regulation (Preston and Notarianni, 1983), possibly by altering the conformation of ICP4 (Blaho et al., 1992).

ICP4 is also nucleotidylated (guanylated and adenylated). These modifications have also been hypothesized to regulate ICP4 transcription activation and HSV-1 replication (Blaho and Roizman, 1991; Blaho et al., 1993).

1.3.2. Phosphorylation of ICP4

ICP4 was originally shown to be phosphorylated by Honess and Roizman (1975), who observed that a shift in the ICP4 band in SDS-PAGE was coincidental with its phosphorylation and translocation to the nucleus. Pereira *et al.*, (1977) found soon after that ICP4 existed in three phosphorylated forms. One form is stably phosphorylated, whereas the other two rapidly cycle (Wilcox et al., 1980). More recently, ICP4 has been further resolved into five phosphorylated forms by two-dimensional analyses, in which

proteins are separated by molecular weight and pH. The most hyperphosphorylated form was inhibited by roscovitine (Rosco), an inhibitor of cyclin-dependent kinases (Advani et al., 2001).

Native ICP4 isolated from HSV-1 infected cells is a homodimer phosphorylated at several serine and threonine residues (Metzler and Wilcox, 1985; Faber and Wilcox, 1986b). Phosphorylation occurs mostly in residues in between positions 170 and 250, which include the previously described serine rich region at the N-terminus of the protein. ICP4 is also phosphorylated in other residues carboxy terminal to position 309 (DeLuca and Schaffer, 1988). Phosphorylation of multiple sites on ICP4 may be sequentially regulated (Xia et al., 1996a).

cAMP dependent protein kinase A (PKA) was suggested to be a major player in phosphorylation of the serine-rich domain of ICP4 (Mullen et al., 1995; Xia et al., 1996a; Xia et al., 1996b). The serine rich tract consists of repeats of 35 residues. Each repeat contains nineteen serines and one threonine flanked by seven basic amino acids at the N-terminus and eight acidic amino acids at the C-terminus **(Figure 1.1)** (Xia et al., 1996b). Consensus sites for protein kinase C (PKC) and casein kinase II (CKII) are also found within this domain (Xia et al., 1996a; Xia et al., 1996b). CKII phosphorylation sites generally consist of a run of glutamate or aspartate residues following the targeted serines or threonines (Edelman et al., 1987; Pearson and Kemp, 1991). The consensus CKII motif is in the C-terminal flanking sequences of the serine rich domain (Xia et al., 1996b). Two PKA phosphorylation sites are found within the N-terminal flanking sequences of the serine-rich domain (Xia et al., 1996b). PKA phosphorylation sites

typically contain a cluster of arginines followed by the targeted serine or threonine residues (Kemp et al., 1977; Pearson and Kemp, 1991).

Xia *et al.* (1996b) suggested that the serine tract is a functional target of PKA and that the interaction between PKA and ICP4 is important for wild-type levels of HSV-1 replication. They also suggested that the serine-rich region of ICP4 stimulates phosphorylation in the rest of the protein. This domain may also regulate the conformation of the rest of the protein. ICP27 has been suggested to modulate the levels of the protein kinases or phosphatases that affect phosphorylation of ICP4 since ICP27 HSV-1 mutants had altered ICP4 phosphorylation patterns (Xia et al., 1996a).

Advani *et al.*, (2001) have suggested that ICP4 is phosphorylated by CDK1. In support of this model, Rosco inhibited phosphorylation of the most hyperphosphorylated form of ICP4 at 10 hours after infection. CDKs phosphorylate peptides containing the consensus sequence (S/T)PX(R/K) where X is any amino acid. Since CDK1 is mostly a cytoplasmic protein, it would have to phosphorylate ICP4 before the protein enters the nucleus.

Deletion of residues 170-250 leads to a decrease in the ability of ICP4 to transactivate transcription of E and L genes (Xia et al., 1996a; Xia et al., 1996b). Deletion of amino acids 162 to 229 also decreases its ability to transactivate transcription of the L glycoprotein gD gene, even in the presence of ICP0 (Paterson and Everett, 1988a).

Phosphorylation of ICP4 does not regulate its ability to form high affinity complexes with its high affinity DNA sites present in IE genes (Papavassiliou et al.,

1991). The effects of phosphorylation in ICP4 subnuclear localization have not been analyzed.

1.4. Cellular and viral nuclear domains during HSV-1 infection

1.4.1. Cellular morphological changes during HSV-1 infection

There are a number of morphological changes that occur within a cell productively infected with HSV-1. Many of these changes occur in the nucleus. The changes in the nucleus include margination of host chromatin, disaggregation of the nucleolus, and the appearance of viral inclusions, among others (Sirtori and Bosisio-Bestetti, 1967; Schwartz, 1969; Wagner and Roizman, 1969; Puvion-Dutilleul et al., 1982; Dargan and Subak-Sharpe, 1983). Some of these changes, such as the margination of host chromatin may be a consequence to the development and enlargement of HSV-1 replication compartments within the cell nucleus.

1.4.2. Nuclear dots 10 (ND10s)

HSV-1 replicates in a limited number of nonrandomly distributed nuclear sites (Quinlan et al., 1984; de Bruyn Kops and Knipe, 1994). These replication sites are thought to be formed by localization of infecting HSV-1 genomes to preexisting nuclear sites, which are adjacent to nuclear structures called nuclear dots 10 (ND10), promyelocytic leukemia (PML) bodies, or promyelocytic oncogenic domains (PODs). ND10s are nuclear matrix-bound structures present with an average frequency of 5 to 20 per nucleus, depending on cell type, and which have a diameter of 0.3 to 0.5 μm in most cells (Ascoli and Maul, 1991). ND10s are devoid of chromatin and nascent RNA, but

newly synthesized RNA associates with their periphery. A subset of ND10 proteins are also found in centromeres in a cell cycle dependent manner (particularly in G2-phase). This localization is augmented by inhibition of the proteasome-mediated proteolysis (Everett et al., 1999a; Everett et al., 1999b).

It is thought that ND10s may contribute to the formation of a favorable local environment for the expression of specific genes (Boisvert et al., 2000). Such an environment would be favorable for HSV-1 transcription too. ND10s have also been suggested to be involved in antiviral defense mechanisms, in that proteins associated with ND10s are upregulated by interferon (Guldner et al., 1992; Korioth et al., 1995; Maul et al., 1995; Chee et al., 2003).

ND10s undergo rapid changes in morphology, composition, and number in response to stresses such as viral infection, DNA damage, heat shock, and treatment with heavy metals, alkylating agents, or drugs that inhibit phosphatases, proteasome, or transcription (Maul et al., 1995; Everett et al., 1999c; Maul et al., 2000; Everett, 2001; Mattsson et al., 2001; Carbone et al., 2002; Barr et al., 2003; Nefkens et al., 2003; Conlan et al., 2004; Eskiw et al., 2004). Within an hour of HSV-1 infection, ICP0 induces proteasome-specific degradation of two essential ND10 proteins, PML and Sp100 (Burkham et al., 1998; Everett et al., 1998; Chelbi-Alix and de The, 1999; Parkinson and Everett, 2000; Everett and Zafiropoulos, 2004). The disruption of ND10s by ICP0 may support the hypothesis that these domains are involved in antiviral mechanisms, in that such disruption has been associated with HSV-1 transcription activation by ICP0 (Davido et al., 2005).

ND10s may also be sites of sequestration of transcription factors and growth regulatory proteins, which would then have to be released to generate an environment conducive to efficient viral gene expression (Burkham et al., 2001). Alternatively, ND10s may be centers for protein modification cascades that regulate the cell cycle (Schwarz et al., 1998; Everett et al., 1999a; Everett et al., 1999b; Takahashi et al., 1999). ND10s may also be sites of deposition of nuclear proteins for their destruction (Maul, 1998). Yet another possibility is that ND10s may mark sites in the nucleus necessary for the establishment of a productive infection, such as a nuclear matrix attachment site (Maul, 1998). Lastly, a recent model proposes that ND10s may be the sites where HSV-1 DNA circularization occurs, which may facilitate recombination and promote HSV-1 DNA replication (Yao et al., 1997; Yeager et al., 1999).

As it transpires from the previous discussion, the function of ND10s is still not well understood. But the majority of infecting HSV-1 genomes localize adjacent to ND10s prior to viral gene expression, and all HSV-1 transcription and replication appears to occur in these nuclear sites (Ishov and Maul, 1996; Maul et al., 1996; Uprichard and Knipe, 1997; for a review on ND10s see Maul, 1998). New evidence suggests that protein components of ND10s may lead to the assembly of novel ND10-like structures in infected cells, which would be associated with the viral nucleoprotein complexes (Everett and Murray, 2005). Following the degradation of both preexisting ND10 and the novel ND10-like structures by ICP0, viral replication compartments may then develop from the viral nucleoprotein complexes (Everett and Murray, 2005).

Stabilization of ND10s by interferon, arsenic trioxide or proteasomal inhibition prevents the disruption of ND10s during HSV-1 infection (Taylor et al., 2000). These

treatments also inhibit formation of RCs and production of progeny virus (Burkham et al., 2001). Stabilization of ND10s therefore correlates with inhibition of viral replication. However, these experiments have not addressed whether stabilization of ND10s inhibits HSV-1 replication or vice-versa.

HSV-1 RCs still form adjacent to ND10s when disruption of ND10s is prevented by using ICP0 null mutants (Maul et al., 1996). Various PML isoforms behave differently during infection in respect to their localization. It is possible that some ND10 proteins may be dispersed under certain conditions while others remain associated in ND10s (Burkham et al., 2001). The compounds used for ND10 stabilization may also prevent the formation of ND10-like structures at viral nucleoprotein complexes. These treatments also tend to inhibit expression of HSV-1 proteins. Thus, the prevention of formation of RCs may be secondary to the effects on HSV-1 replication.

1.4.3. Replication compartments (RCs) are the sites of HSV-1 DNA replication and transcription

1.4.3.1. Cellular "replisomes"

The cellular "replisomes" are nuclear structures thought to contain DNA polymerase alpha and delta, DNA primase, topoisomerases I and II, RNase H, proliferating cell nuclear antigen (PCNA), a DNA-dependent ATPase, replication factor C, DNA ligase I, DNA helicase, and replication protein A (RPA) (Applegren et al., 1995). The "replisomes" have been shown to be attached to the nucleoskeleton (Hozak et al., 1993), and appear to increase in size but decrease in number during progression through S phase (Hozak et al., 1994; for review on "replisomes" see Frouin et al., 2003).

The "replisomes" are also sites of cellular transcription (Hassan and Cook, 1994). However, transcription and replication don't occur at the same time within these sites (Wansink et al., 1994; for review see Cook, 1999).

Many of the proteins in the cellular "replisomes" are regulated by the cellular cyclin-dependent kinases (CDKs). For example, CDK2/cyclin A phosphorylates RPA which inhibits DNA synthesis (Cardoso et al., 1993; for review see Henneke et al., 2003), and CDK2/cyclin E phosphorylates DNA polymerase alpha which stimulates progression of DNA synthesis (for review see Henneke et al., 2003).

1.4.3.2. RCs and their precursors

^{3}H-thymidine labeling of HSV-1 infected cells demonstrated that HSV-1 DNA synthesis is restricted to well defined nuclear sites, which increase in size as the infection proceeds (Roizman, 1969; Rixon et al., 1983). These sites were shown early on to contain ICP4, ICP8, and the viral DNA polymerase (Randall and Dinwoodie, 1986). They were later shown to be the sites in which HSV-1 genomes are replicated and transcribed (de Bruyn Kops and Knipe, 1988; Rice, 1994; Leopardi et al., 1997; Phelan et al., 1997).

RCs form adjacent to preexisting nuclear structures, in that RCs localized symmetrically in binucleated cells (de Bruyn Kops and Knipe, 1994). The RCs are thought to form at the cellular "replisomes" sites located adjacent to the ND10s (Zhong and Hayward, 1997).

RCs are the sites in which HSV-1 DNA replication occurs. They are thought to be formed from the pre-replication compartments (pre-RCs) in which HSV-1 DNA

replication does not occur. In turn, the pre-RCs are thought to be formed from the pre-replication foci (pre-RFs). Pre-RFs are thought thus to be the first viral compartments formed at the cellular "replisomes". These pre-RFs include the HSV-1 helicase-primase complex (UL5, UL8, UL52) and the ssDNA binding protein (ICP8). The pre-RCs contain the "core" pre-RF proteins, plus the origin binding protein (UL9), viral DNA polymerase (UL30), processivity factor (UL42), ICP4, and ICP27. The RCs contain all the components of the pre-RC, together with HSV-1 genomes.

RCs were originally defined as numerous small nuclear punctae of accumulation of ICP8 protein (Quinlan et al., 1984), which was already known to associate with replicating HSV-1 DNA (Powell and Purifoy, 1976; Knipe and Spang, 1982). ICP8 thus localizes to the sites of HSV-1 DNA replication, where it binds to single-stranded HSV-1 DNA at the replication fork (Lee and Knipe, 1983; Puvion-Dutilleul et al., 1985). However, most of the so-defined pre-RCs contain no HSV-1 genomes. And most HSV-1 DNA is not found at ICP8-defined sites but rather at preexisting sites adjacent to ND10s (Maul et al., 1996; Sourvinos and Everett, 2002). Only the pre-RCs located near ND10s do contain HSV-1 genomes. Therefore, not all small pre-RCs defined by accumulation of ICP8 are potential future replication sites (Maul et al., 1996) and the HSV-1 genomes that accumulate at ND10s have an increased probability of initiating HSV-1 DNA replication. According to these newer results, thus, accumulation of ICP8 does not define the functional pre-RC.

HSV-1 amplicons are small HSV-1-derived circular DNA minimally containing the origin of replication and "a" sequences required for cleavage and packaging. HSV-1 amplicons are replicated and packaged in the presence of "helper virus" which provides

in *trans* all HSV-1 proteins required for replication and packaging. HSV-1 amplicons have been used to study the formation of RCs. Tang *et al.* found that the IE proteins ICP4 and ICP27, as well as an HSV-1 origin of replication, were required for efficient transcription of amplicons (Tang et al., 2003). The amplicons localized to ND10s via binding to ICP4 and ICP27, which both in turn interacted with Daxx, a protein associated with ND10s.

The localization of ICP4, ICP27, and HSV-1 genomes adjacent to ND10s was later confirmed using live cell imaging (Everett et al., 2004). ICP4 and ICP27 were also shown to colocalize to ICP0 containing punctae and ICP0 was suggested to be a major determinator of this localization (Mullen et al., 1995). Most of the foci containing ICP4 associated with ICP0 and adjacent to ND10s later developed into RCs, therefore suggesting that most of these foci also contain HSV-1 genomes (Everett et al., 2003).

RCs are located in the interior of the cell nucleus (de Bruyn Kops and Knipe, 1994). This localization is consistent with older results, which demonstrated that chromatin was displaced to the periphery of the nucleus at 3-4 hours post-infection (hpi) (Randall and Dinwoodie, 1986; Knipe, 1989).

1.4.3.3. Formation of RCs

One of the models proposed for the formation of replication compartments postulates that the first structures formed are the pre-RFs. These foci are thought to represent a reorganization of cellular "replisomes", together with accumulation of the viral helicase-primase complex (UL5, UL8, UL52) and the ssDNA binding protein (ICP8) (Quinlan and Knipe, 1985; de Bruyn Kops and Knipe, 1988; Zhong and Hayward,

1997). Pre-RCs are then formed with UL5, UL8, UL52, and ICP8 as the core proteins (Goodrich et al., 1990; Bush et al., 1991; Calder et al., 1992; Liptak et al., 1996; Lukonis and Weller, 1996). UL5, UL8, UL52, and UL9 are necessary for the localization of ICP8 to pre-RCs. This suggests that the origin-binding protein UL9 localizes to the pre-RC sites almost simultaneously with the core proteins themselves (Liptak et al., 1996). UL42 then localizes to the pre-RCs and facilitates the recruitment of UL30 (Liptak et al., 1996). ICP4 and ICP27 also localize to the pre-replication foci to form the pre-replication compartments (Rixon et al., 1983; Knipe and Smith, 1986; Zhong and Hayward, 1997). Direct protein-protein interactions between ICP8 and UL30, ICP8 and UL9, or UL8 and UL9 are probably important in the recruitment of these proteins to pre-RFs to form pre-RCs (Ruyechan and Weir, 1984; Chiou et al., 1985; O'Donnell et al., 1987; Bush et al., 1991; Boehmer and Lehman, 1993; Boehmer et al., 1994; McLean et al., 1994; Zhong and Hayward, 1997). However, UL9 does not completely localize with ICP8 into pre-RCs, and ICP4 does not localize to pre-RCs in the presence of PAA (Randall and Dinwoodie, 1986; Knipe et al., 1987; Malik et al., 1996).

Pre-RCs can also form without UL42 or UL30. In the latter case, PML also failed to localize to these compartments (Burkham et al., 1998). Full RCs are thought to form only after all seven of the HSV-1 DNA replication proteins, ICP4, ICP27 (Mullen et al., 1994; Phelan et al., 1997), and the HSV-1 genomes localize to the RCs. Once RCs are formed, HSV-1 replication occurs (Zhong and Hayward, 1997) **(Figures 1.2 and 1.3)**.

ICP27 is thought to promote HSV-1 DNA replication in that ICP27 null mutants produce only between 8-23% of the DNA amounts produced by wild-type virus (Sacks et al., 1985; McCarthy et al., 1989; Rice et al., 1989; Rice and Knipe, 1990). Furthermore,

proper ICP8 distribution within RCs was found to be disrupted in cells infected with ICP27 null mutant virus. This redistribution of ICP8 could potentially result from a change in its conformation in the absence of ICP27 (Curtin and Knipe, 1993). ICP27 deletion or nonsense mutants also show reduced levels of accumulated mRNA of UL5, UL8, UL9, UL42, UL52, and UL30, which could then lead to an indirect inhibition of HSV-1 DNA synthesis (Uprichard and Knipe, 1996).

Several cellular proteins have also been found to accumulate to RCs, including the tumor suppressors or cell cycle regulators pRb and p53, and several proteins that regulate cellular DNA replication, such as DNA polymerase delta and DNA ligase (Wilcock and Lane, 1991). The accumulation of cellular cell cycle regulators in these compartments suggests that a specific subset of cell cycle regulators may play important roles during HSV-1 infection.

It is not clearly understood yet how ICP4 is recruited to RCs. Two mechanisms are most commonly considered. In one model, ICP4 is recruited into pre-RCs and RCs by the ICP4 binding sites in the HSV-1 genomes which have accumulated in these compartments (Everett et al., 2004). In the other model, ICP4 is recruited by binding to proteins at the core of the pre-RCs or RCs (Zhong and Hayward, 1997).

1.4.3.4. Studies on the mechanisms of formation of RCs

It is hypothesized that there are two populations of pre-RCs in the presence of PAA (Lukonis et al., 1997; Uprichard and Knipe, 1997). One population is S-phase dependent and colocalize with sites of cellular DNA synthesis. The other population is S-phase independent and form adjacent to ND10s (Uprichard and Knipe, 1997). It is

most likely that the S-phase independent pre-RCs are the precursors for full RCs, as they occur at the sites where the incoming viral genome and viral proteins accumulate (Ishov and Maul, 1996; Maul et al., 1996; Lukonis et al., 1997; Uprichard and Knipe, 1997).

de Bruyn Kops and Knipe (1988) had suggested that ICP8 is the major organizational protein of RCs, recruiting all other viral and cellular proteins. The processivity factor UL42 localizes to RCs together with ICP8 and the viral DNA polymerase. However, ICP8 and a percentage of viral DNA polymerase still localize to small pre-RC in the presence of PAA, conditions in which UL42 localizes diffusely throughout the nucleus. Therefore, ICP8 is not sufficient to recruit UL42 to pre-RCs (Goodrich et al., 1990). de Bruyn Kops *et al.* (1998) also showed that ICP8 and UL30 localize to a punctuate distribution within RCs, but that they relocalize to pre-RCs when when PAA was added to cells containing preformed RCs. In contrast, ICP4 and ICP27 showed a more diffuse distribution within RCs, which was not affected when PAA was added to cells containing preformed RCs. In contrast, ICP4 does not localize to pre-RCs if PAA is added to the infected cells before the formation of the pre-RCs (Randall and Dinwoodie, 1986; Knipe et al., 1987).

Certain host recombination proteins such as RPA, RAD51, and NBS1 localize to the pre-RCs adjacent to ND10s (Yeager et al., 1999; Wilkinson and Weller, 2004). This localization is dependent on the presence of the origin binding protein UL9 and HSV-1 DNA polymerase UL30, as well as on the integrity of ND10s (Wilkinson and Weller, 2004).

The capsid protein VP5 also localizes to RCs, but at later times post-infection (McNabb and Courtney, 1992; Church and Wilson, 1997; Lamberti and Weller, 1998).

VP5 is thought to be first distributed diffusely throughout the nucleus and RCs, and then to punctuate structures within RCs (de Bruyn Kops et al., 1998). Another capsid protein, VP19c was also shown to colocalize with ICP8 to RCs at late times post-infection (Chowdhury and Batterson, 1994). It was hypothesized that the packaging protein UL32 may play a role in recruiting preassembled capsids to the sites of DNA packaging, which would be within or adjacent to RCs. This recruitment would then allow cleavage and packaging of viral DNA (Lamberti and Weller, 1998).

A subset of ICP0 is also thought to localize to RCs (Knipe et al., 1987), as is ICP22 phosphorylated by UL13 and U_S3. ICP22 thus colocalizes with ICP4, RNAPII, host nucleolar "EBER-associated protein" (EAP), and viral DNA (Leopardi et al., 1997). McNamee *et al.* found that ICP8 deleted in amino acid residues 1082-1169 failed to localize to RCs and further prevented localization of wild type ICP8 and other proteins (McNamee et al., 2000). This mutant also prevented HSV-1 DNA replication. Inhibition of DNA replication may have been secondary to the inhibition of the proper nuclear localization of ICP8. Or inhibition of the proper localization of ICP8 may have been secondary to the inhibition of DNA replication. The deleted C-terminal region of ICP8 contains two alpha-helices at amino acid residues 1080-1135, which are thought to be required for proper localization of ICP8 to pre-RCs and RCs, as analyzed by proline substitutions (Taylor and Knipe, 2003).

ICP8 localizes to pre-RCs prior to HSV-1 DNA replication, and it also binds to the nuclear matrix. ICP8 still binds to the nuclear matrix when it is also bound to viral DNA (Lee and Knipe, 1983; Quinlan and Knipe, 1983). Thus, HSV-1 DNA may be indirectly bound to the nuclear matrix by ICP8 (Quinlan and Knipe, 1983). This

interaction between ICP8 and the nuclear matrix is independent of HSV-1 DNA replication, in that it is not disrupted by PAA (Lee and Knipe, 1983; Quinlan and Knipe, 1983).

It was suggested that ICP8 has two functional sites, one required for the localization to the pre-RCs and one for binding DNA (Lee and Knipe, 1983; Ruyechan and Weir, 1984; Chiou et al., 1985; O'Donnell et al., 1987; Wang and Hall, 1990; Bush et al., 1991; Boehmer and Lehman, 1993; Boehmer et al., 1994; McLean et al., 1994). After HSV-1 DNA synthesis starts, ICP8 localizes into large RCs where it also binds HSV-1 DNA. ICP8 molecules may move from one nuclear location (pre-RCs) to the other (RCs) upon initiation of viral DNA synthesis. Under this model, pre-RCs serve as a reservoir for "free" ICP8 molecules (i.e., not bound to replicating or progeny HSV-1 DNA). Alternatively, the different nuclear sites of nuclear association contain ICP8 in different stages of maturation (Quinlan et al., 1984).

Certain temperature-sensitive mutant forms of ICP4 prevent ICP8 and ICP0 localization to the nucleus (Knipe and Smith, 1986). However, ICP8 and ICP0 localized to the nucleus in transfections in the absence of ICP4. Mutant ICP4 may thus block binding of ICP8 and ICP0 to the nuclear matrix or to other proteins required for their recruitment (Knipe and Smith, 1986).

de Bruyn Kops (1988) proposed a model in which cellular DNA replication complexes formed prior to infection are incorporated into the viral pre-RCs and provide the cellular factors required for efficient HSV-1 DNA replication. They also hypothesized that ICP8 plays a direct role in the reorganization of the cell nucleus, or in the maintenance of the reorganized state. ICP8 localizes adjacent to ND10s and therefore

may play a role in directing RCs to these nuclear matrix-bound structures (Lukonis and Weller, 1997).

Formation of full RCs from pre-RCs has been hypothesized to occur by two mechanisms. Pre-RCs may move to coalesce and form larger compartments. Alternatively, pre-RCs may expand while maintaining the same relative position within the nucleus. Larger compartments would then be formed once the boundaries of adjacent compartments merge (Taylor et al., 2003). The movement of pre-RCs to form RCs could be mediated by actin or myosin motors (Taylor et al., 2003), but these theories remain to be tested. Alternatively, the ability of herpesviruses to disrupt the nuclear lamina (an important component of the nuclear matrix) may allow for the movement of viral replication structures (Scott and O'Hare, 2001; Muranyi et al., 2002). It has been suggested that the mobility of most nuclear proteins is energy independent and, therefore, likely to occur by diffusion-based, passive, nondirected mechanism (Pederson, 2000; Phair and Misteli, 2000; Shopland and Lawrence, 2000; Misteli, 2001). However, the mechanisms of nuclear movement of HSV-1 proteins remain unknown.

RC maturation also involves the marginalization of the host chromatin (Monier et al., 2000). RCs then expand to approach the nuclear envelope (Roller et al., 2000; Reynolds et al., 2001; Reynolds et al., 2002), reorganization that is coupled with a two-fold increase in nuclear volume (Monier et al., 2000). It is thought that the products of UL31 and UL34 may induce the redistribution of lamin A/C and lamin-associated protein 2 (Simpson-Holley et al., 2004). UL31 and UL34 proteins are also thought to mediate the redistribution of lamin B receptor (LBR) and the decrease in lamin A/C and B (Scott

and O'Hare, 2001). This disruption of the nuclear lamina and host chromatin would therefore allow viral capsids to exit the nucleus, and eventually egress the cell.

1.5. The cell cycle in eukaryotic cells

1.5.1. Introduction to the cell cycle and its regulation

The cell cycle includes four major phases: Gap1 (G1), DNA Synthesis (S), Gap2 (G2), and Mitosis (M). G1 involves the preparation to DNA synthesis and is the longest phase. It typically lasts more than 10h, although the actual times depend on cell type and culture condition. S-phase lasts 6 to 8h and is the period when DNA is replicated. G2-phase lasts 2 to 6h and involves the preparation of the cell for mitosis. M-phase is mitosis, or cell division, and is the shortest, lasting only 1h. Lastly, cells may also enter a quiescent state called G0.

G1-phase is when the cell prepares itself for DNA replication. The cell commits to progress into S-phase or to leave the cell cycle and enter the resting phase, G0. When the cell has reached a threshold size and has past the restriction point (when it becomes irreversibly committed to progress through the cell cycle) it enters into S-phase. Following DNA replication, cells enter G2-phase, during which they duplicate in size. When they have reached a threshold size, cells undergo mitosis (or enter M-phase), during which the mother cell generates two identical daughter cells. Once mitosis is completed, the cell cycle starts anew with a new G1-phase (for review see Murray, 1994).

1.5.2. Cyclin-dependent kinases (CDKs) are major regulators of the cell cycle

The highly conserved serine/threonine protein kinases known as cyclin-dependent kinases (CDKs) are major regulators of the cell cycle (for review see Morgan, 1997). Active CDKs are composed of a catalytic subunit, the CDK, and a regulatory subunit, a cyclin. Each CDK binds to specific cyclin partners, which may mediate in part their substrate specificity (Higashi et al., 1995). Whereas the CDKs are constitutively expressed, the cyclins are differentially expressed during the cell cycle.

The D-type cyclins (cyclin D1, D2, and D3) form complexes with CDK4 and CDK6, complexes which are most active in early to middle G1-phase. CDK4/cyclin D or CDK6/cyclin D complexes mediate the phosphorylation of the retinoblastoma (pRb) protein from the hypophosphorylated to the hyperphosphorylated form. This phosphorylation partially inhibits the ability of pRb/E2F complexes to negatively regulate the transcription of genes required for S-phase entry. Hyperphosphorylated pRb releases the transcription and cellular replication promoting activities of E2F (for review see Sherr, 1995; Trimarchi and Lees, 2002). More recent studies have implicated histone deacetylases (HDACs) and the human SWI-SNF complex as required for pRB repression (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998; Zhang et al., 2000). In this case, pRB is thought to recruit HDAC and SWI-SNF to the promoter, whereby these enzymes alter the chromatin structure to a repressed state. The requirement for HDAC activity appears to be limited to a subset of promoters, and recent *in vivo* experiments suggest that repression by pRB occurs through both HDAC-dependent and -independent mechanisms (Luo et al., 1998; Chen and Wang, 2000; Dahiya et al., 2000). Inactivation of cyclin D1 via withdrawal of extracellular mitogens prior to the restriction point blocks progression into S-phase. However, inactivation beyond the restriction point

has no effect (Sherr, 1994), and cyclin D1 null cells do not arrest in G1 (Fantl et al., 1995; Sicinski et al., 1995; Sicinski et al., 1996).

CDK2 is activated by cyclin E at late G1 triggering DNA synthesis (S-phase) (for review see Lee and Yang, 2003). CDK2 in complexes with cyclin A further promotes S-phase progression. CDK1 in complexes with cyclins B or A promotes G2/M transition to initiate mitosis. The exit from mitosis depends on the ubiquitin-mediated degradation of cyclin B and the consequent inactivation of CDK1.

To be active, CDKs must be bound to a proper cyclin subunit. But full CDK activation only occurs when the CDK activating kinase (CAK - which itself consists of CDK7/cyclin H/MAT1) phosphorylates the CDK on a conserved threonine residue located in the so-called "T" loop (Poon et al., 1993). Other protein kinases such as *wee-1/mik*-1 inactivate CDK/cyclin complexes through phosphorylation of other threonine or tyrosine residues located near the N-terminus. The inactive phosphorylated CDKs can then be activated through dephosphorylation by the cdc25C phosphatase.

Active CDK complexes are further regulated by the CDK inhibitors (CKIs). CKIs are divided in two groups. The first group includes the broad inhibitors that associate with CDK/cyclin complexes and proliferating cell nuclear antigen (PCNA). This group includes p21 and $p27^{Kip1}$, which may also be mediators of extracellular antimitogenic signals (Polyak et al., 1994). The second group, which includes p16, includes proteins that are more restricted in their ability to inhibit CDK activities. These proteins are competitive inhibitors of binding of CDK4 or CDK6 to D-type cyclins. The CDK/cyclin complexes are also affected by subcellular localization and ubiquitin-mediated degradation of cyclins. All levels of regulation modulate progression through

the cell cycle (reviewed in Murray, 1994; Beijersbergen and Bernards, 1996; Morgan, 1997).

1.5.3. Cell cycle regulatory CDKs and cyclins

Disruption of mouse CDK4 (Rane et al., 1999; Tsutsui et al., 1999), CDK6 (Malumbres et al., 2004), cyclin D1, D2 (Fantl et al., 1995; Sicinski et al., 1995; Sicinski et al., 1996), D3 (Sicinska et al., 2003), B2 (Brandeis et al., 1998), A1 (Liu et al., 1998), E1, or E2 (Geng et al., 2003; Parisi et al., 2003) does not result in death. However, disruption of these CDKs or cyclins results in other, subtler phenotypes, such as growth retardation, infertility, and diabetes in CDK4 -/- mice (Rane et al., 1999; Tsutsui et al., 1999), or infertility in cyclin D2 -/- mice (Sicinski et al., 1996). CDK 6 -/- mice show mild defects in defined hematopoietic cell populations (resulting from decrease in size of thymus and spleen) (Malumbres et al., 2004). Cyclin D1 -/- mice show growth retardation and neurological disorders (Sicinski et al., 1995) as well as impaired eye and mammary gland development (Fantl et al., 1995; Sicinski et al., 1995). Cyclin D3 -/- mice fail to undergo normal expansion of immature T lymphocytes and show reduced sensitivity to T cell malignancies (Sicinska et al., 2003). Cyclin A1 -/- mice show sterility in males (Liu et al., 1998), and cyclin E1 -/- and cyclin E2 -/- mice show placental and cardiac defects (Geng et al., 2003; Parisi et al., 2003). No developmental abnormalities have been reported in cyclin B2 -/- mice (Brandeis et al., 1998). In contrast, disruption of cyclin A2 or cyclin B1 causes embryonic lethality (Murphy et al., 1997; Brandeis et al., 1998), demonstrating that these cyclins are essential for mouse development (for review on CDKs and cyclins see Sherr and Roberts, 2004).

The importance of CDK2 in the cell cycle has been well documented. For example, catalytically inactive CDK2 mutants induce a G1 block (van den Heuvel and Harlow, 1993). "Dominant negative" CDK2 mutants are catalytically inactive CDK2 mutants. When overexpressed, dominant negative CDK2 bind to cyclin A and E and thus prevent binding of the wild-type CDK2. Consequently, dominant negative CDK2 mutants prevent activation of the endogenous CDK2.

Overexpression of the CDK2 inhibitor $p27^{Kip1}$ or microinjection of antibodies against CDK2, cyclin E, or cyclin A causes cell cycle arrest at G1/S and blocks initiation of DNA synthesis (Pagano et al., 1992; Polyak et al., 1994; Ohtsubo et al., 1995). CDK2 was shown to be involved in entry into mitosis in other human cells (Hu et al., 2001). Overexpression of a dominant negative CDK2 was found to cause cell cycle arrest in G1 in U2OS cancer cell lines (van den Heuvel and Harlow, 1993). However, dominant negative CDK2, antisense oligonuleotides to CDK2, or overexpression of the CKI, $p27^{Kip1}$ did not arrest human U2OS cancer cell lines in a more recent study (Tetsu and McCormick, 2003). Even more surprising, CDK2 -/- mice are viable, although they are sterile and somewhat smaller. These results suggest that CDK2 is required for meiotic, but not for mitotic, cell cycles (Berthet et al., 2003).

CDK3 may be an alternate kinase subunit for cyclin E2 (Zariwala et al., 1998). CDK3 is highly homologous to CDK1 and CDK2. However, CDK3 cannot compensate for the loss of CDK2 in certain strains of laboratory mice that have a point mutation in CDK3 that creates a null allele (Ye et al., 2001). "Wild-type" mice (*Mus spretus* and *Mus mus castaneus)* lack this mutation (Ye et al., 2001). CDK1 may also bind to cyclin E to regulate G1/S phase transition in the absence of CDK2 (van den Heuvel and Harlow,

1993; Aleem et al., 2005). However, cyclin E1 and E2 are both also dispensable *in vivo* (Geng et al., 2003; Parisi et al., 2003). Alternatively, cyclin A2, which is essential for viability, may also bind CDK1 to activate cell cycle progression (Roberts and Sherr, 2003).

1.5.1. Other CDKs and cyclins

Other CDKs are not exclusively involved in the cell cycle. For example, CDK7, 8, and 9 are involved in transcription. CDK7 and cyclin H form a complex together with ménage à trois 1 (MAT1), which is known as the CDK-activating kinase (CAK). CAK activates CDK1, CDK2, CDK4, and CDK6 by phosphorylation of a specific threonine residue in the "T" loop (Solomon et al., 1992; Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993; Fisher and Morgan, 1994; Kato et al., 1994; Matsuoka et al., 1994). CAK, however, is also found as a component of TFIIH, which includes DNA helicase and transcription/repair activities (for review on TFIIH see Svejstrup et al., 1996). CDK7 in TFIIH phosphorylates the CTD of RNAPII to allow promoter clearance, thus initiating transcription (Lu et al., 1992; Goodrich and Tjian, 1994; Dahmus, 1996; Svejstrup et al., 1996; Oelgeschlager, 2002; Palancade and Bensaude, 2003; Shilatifard et al., 2003; for review see Lolli and Johnson, 2005). CDK7 itself can be phosphorylated by CDK1/cyclin B and CDK2/cyclin A, but this phosphorylation is not essential for CAK activity (Garrett et al., 2001).

In contrast to CDK7, CDK8/cyclin C inhibits transcription by phosphorylating the CTD of RNAPII before the polymerase binds to the promoter to form the pre-initiation complex. This effect is promoter specific (reviewed in Bregman et al., 2000).

CDK9/cyclin T1, known as P-TEFb, further phosphorylates the CTD of RNAPII to foster transcription elongation and processivity (reviewed in Bregman et al., 2000).

CDK5 is a unique CDK in that it is involved in neural differentiation, but not in cell cycle regulation (Ohshima et al., 1996; Chae et al., 1997). CDK5 is also unique in that it is activated by binding to a non-cyclin, and neuron-specific, protein, p35 (Lew et al., 1994; Tsai et al., 1994; Chae et al., 1997). p35 -/- or CDK5 -/- mice display cortical lamination defects, seizures, and spontaneous perinateal and adult mortality (Ohshima et al., 1996; Chae et al., 1997).

1.6. Pharmacological cyclin-dependent kinase inhibitors (PCIs)

1.6.1. Introduction

CDKs are critical in cell proliferation, transcription, neuronal functions, cell death, and other functions. These roles suggested the potential application of pharmacological CDK inhibitors (PCIs) in cancers, cardiovascular diseases, glomerulonephritis, and diseases affecting the nervous system, as well as for *in vitro* fertilization. It was later suggested that these PCIs could also be useful against microbes that use CDKs for their replication.

PCIs are a diverse group of relatively specific CDK inhibitors, which despite chemical diversity share many common properties. PCIs are flat, hydrophobic heterocycles of low molecular weight (<600 Da) **(Figure 1.4.)**. Most PCIs are competitive inhibitors (with respect to ATP). They commonly bind to the ATP binding pocket by mostly hydrophobic interactions and a limited number of hydrogen bonds (Knockaert et al., 2002).

1.6.2. Flavopiridol (Flavo)

(-)-cis-5,7-dihydroxyphenyl-8-[4-(3-hydroxy-1-methyl)piperidinyl]-4H-1-benzopyran-4-one (L868276) is a flavone, which in its chlorinated form is named flavopiridol (Flavo). The chlorophenyl group in Flavo, which replaces a phenyl group in L868276, increases the CDK inhibitory potency by a factor of 6 (De Azevedo et al., 1996). Flavo is a pan-specific PCI that preferentially inhibits CDK 1 (IC_{50} 0.40 μM), CDK2 (IC_{50} 0.28 μM), CDK4 (IC_{50} 0.40 μM), CDK6, and CDK9 (Losiewicz et al., 1994; Carlson et al., 1996a; Carlson et al., 1996b; Sedlacek, 1996; Chao et al., 2000). Flavo inhibits CDK9 non-competitively (Chao et al., 2000). In culture, it causes a G1 or G2 arrest thought to result from the inhibition of the kinase activities of CDK1, CDK2, and CDK4. However, it is now known that CDK2 and CDK4 are not required for cell cycle progression. CDK4 inhibition indirectly inhibits hypophosphorylation of pRb (Losiewicz et al., 1994; Carlson et al., 1996b; Sedlacek, 1996). Flavo also causes a global reduction in mRNA levels, an effect that may be a major contributor to its cytotoxicity (Lam et al., 2001). This reduction in mRNA levels may also negatively affect the cell cycle due to low cyclin levels.

In a co-crystal structure with CDK2, the aromatic ring of L868276 was found in the ATP-binding site of CDK2. However, the position of the phenyl group of L868276 enables it to make contacts with CDK2 that are not present in the ATP-CDK2 complex. Binding is characterized primarily by hydrophobic and van der Waals interactions with the hydrophobic residues that form the ATP pocket. L868276 also fits very well in the ATP binding pocket of CDK2 (De Azevedo et al., 1996).

Flavo inhibits transcription elongation by RNAPII *in vitro*, a step which is controlled by P-TEFb phosphorylation of the CTD of RNAPII. P-TEFb is especially required for Tat activated HIV-1 transcription elongation. Hence, Flavo inhibited Tat transactivation and HIV-1 replication with an IC_{50} of 10 nM (Chao et al., 2000). Using nuclear run on assays, high concentrations of Flavo (300 nM) inhibited most cellular RNAPII transcription *in vivo* (Chao and Price, 2001). However, Flavo has no effect on cellular gene transcription *in vivo* at concentrations that block HIV replication (Chao and Price, 2001).

1.6.3. Roscovitine (Rosco)

2-(*R*)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine or roscovitine (Rosco) is a purine-PCI. Rosco is a highly potent and selective inhibitor of a subset of CDKs, which was first synthesized by Meijer *et al.* (1997). Rosco is selective for CDK1/cyclin B (IC_{50} 0.65 μM), CDK2/cyclin A (IC_{50} 0.7 μM), CDK2/cyclin B (IC_{50} 0.7 μM), CDK5/p35 (IC_{50} 0.16 μM) (Meijer et al., 1997), CDK7 (IC_{50} 0.45 μM) (Schang et al., 2002a), and CDK9. ProQinase has found that Rosco also inhibits CDK3 activity at an IC_{50} of 1.4/1.5 μM (Bach et al., 2005). At higher concentrations, it inhibits erk1 and 2 (IC_{50} 34 μM and 14 μM, respectively) (Meijer et al., 1997). Rosco also inhibits DYRK1A (IC_{50} 3.1 μM) at concentrations approximately 5-fold above those that inhibit CDK2, and CK1δ (IC_{50} 17 μM) at concentrations approximately 25-fold higher than those that inhibit CDK2 (Bain et al., 2003).

More recently, the specificity of Rosco was further evaluated by analyzing its interactions with 119 different protein kinases (Fabian et al., 2005). The protein kinase

domains of the 119 proteins were expressed as fusions with T7 bacteriophage major capsid protein. The phages were then bound onto streptavidin-coated magnetic beads treated with biotinylated small molecule ligands, and Rosco in solution was used to compete with the tethered ligand. The amount of phage bound to the tethered ligand after incubation with or without Rosco was then quantified to determine the affinity of Rosco for each protein kinase. Rosco bound to only 11 of the 119 tested kinases, including CDK2 and CDK5, which were the only CDKs included in this screen, and the CDK-like kinases CLK1, CKL2, and CKL4 (Fabian et al., 2005).

Since Rosco contains one chiral carbon it exists as two optical isomers. In one report, *R*-Rosco displayed a 2-fold lower IC_{50} for CDK1/cyclin B than *S*-Rosco (IC_{50} 0.45 μM and IC_{50} 0.95 μM, respectively), while the racemic mixture showed intermediate activity (IC_{50} 0.65 μM). However, these differences were non-significant when tested against CDK2/cyclin E (De Azevedo et al., 1997; Meijer et al., 1997).

Rosco is a competitive inhibitor, competing with the ATP co-substrate by binding the ATP-binding pocket of the target CDKs. In a Rosco/CDK2 co-crystal structure, the purine ring of Rosco occupied approximately the same area as the purine ring of ATP, except that the ring of Rosco was rotated by approximately 180° with respect to that of ATP. Furthermore, the benzyl ring of Rosco pointed toward the outside of the ATP-binding pocket, occupying a region not occupied by any atoms of ATP. It is thought that the benzyl ring is the moiety that provides most of the specificity of Rosco. This benzyl ring may also be the structural basis whereby Rosco preferentially inhibits CDK1, CDK2, CDK5, and CDK7 over other CDKs. Like that of Flavo, Rosco binding to CDK2 is also

characterized primarily by hydrophobic and van der Waals interactions (De Azevedo et al., 1997).

Rosco acts on many cell types, but at concentrations 30-100 fold higher than those required to inhibit the purified enzymes. This difference was suggested to be due to poor permeability, unfavorable compartmentalization, presence of unidentified binding proteins, high concentrations of cellular ATP, or possibly a combination of these factors. In contrast, Rosco does not appear to be metabolized to any major extent in cultured cells (Vita et al., 2005).

In a recent study, CDK2 -/- mouse embryonic fibroblasts (MEFs) were exposed to different concentrations (0 – 100 μM) of *R*-Rosco or *S*-Rosco for 96h. CDK2-/- MEFs were only about 1.5-2 fold less sensitive to killing by Rosco than CDK2 +/+ MEFs. These data suggest that CDK2 inhibition accounts for only a fraction of the cytotoxic effects of Rosco, and that other targets are involved (Bach et al., 2005). In contrast, wild-type MEFs accumulated in S-phase in the presence of Rosco, while CDK2 -/- MEFs did not. These latter results suggest that CDK2 is indeed an important target for the effects of Rosco on progression through G1 into S-phase.

Rosco was also found to bind to, and inhibit, one non-protein kinase target, pyridoxal kinase. Pyridoxal kinase is the enzyme responsible for phosphorylation and activation of vitamin B6. Differing from its interactions with CDKs, Rosco interacted with the pyridoxal binding site of pyridoxal kinase, but not with its ATP-binding site (Bach et al., 2005). However, Rosco did not inhibit the kinase activity of pyridoxal kinase efficiently at lower than physiological concentrations of ATP (Bach et al., 2005). It is thus likely that Rosco does not inhibit pyridoxal kinase *in vivo*.

Over 148 proteins (mostly protein kinases) have been tested for their sensitivity to Rosco. Rosco is thus arguably the inhibitor tested against the largest number of potential substrates, even though it has been tested against only 28.6% of the entire human kinome (which consists of 518 kinases) (Bach et al., 2005).

1.7. Effects of Rosco on HSV-1 replication

Rosco inhibits HSV-1 replication, as well as accumulation of IE and E RNAs, with an IC_{50} of 37μM in Vero cells and 10μM in HEL cells. These results suggest that CDKs, or other Rosco-sensitive protein kinases, are required for HSV-1 replication. Rosco-resistant HSV-1 mutants could not be isolated after 11 passages in selective media, further suggesting that CDKs, or other Rosco-sensitive protein kinases, may be required for more than one HSV-1 function (Schang et al., 1998). Rosco also inhibited VP16-dependent activation of IE gene expression, although it did not affect VP16/Oct-1/HCF binding to their target DNA sequence (Jordan et al., 1999). Schang *et al.* have suggested that Rosco likely inhibits HSV-1 and HIV-1 replication by targeting cellular, not viral proteins (Schang et al., 2000). In support of this model, a purine-PCI which is less specific than Rosco, purvalanol, bound to the same proteins in mock- and HSV-1-infected cells, indicating that it did not bind with high-affinity to any viral protein (Schang et al., 2002a).

1.8. Studies on cell cycle regulation during HSV-1 infection

It has been suggested that HSV-1 inhibits multiple phases of the cell cycle via inhibition of various CDKs and cyclins. However, experiments using CDK inhibitors

like Rosco suggest that certain CDKs are actually required for HSV-1 replication. I will discuss below published results dealing with several potential effects of HSV-1 infection on the cell cycle. These results demonstrate the current debate in the field of cell cycle modulation and HSV-1 infection.

It was suggested that HSV-1 infection of cells in G1-phase prevents these cells from entering S-phase (de Bruyn Kops and Knipe, 1988). However, there was no effect on cell cycle progression or HSV-1 replication when the cells were infected in late G1 or S-phase (de Bruyn Kops and Knipe, 1988). Song *et al.* further suggested that HSV-2 inhibits phosphorylation of pRb (Song et al., 2000). In contrast, HSV-2 infection was suggested by another group to lead to an increased phosphorylation of pRb and activation of CDK2 activity, although in the absence of cell cycle progression (Hossain et al., 1997). CDK4 and CDK1 were not activated in these experiments, potentially demonstrating the discrimination of HSV-2 for activation of certain components of the cell cycle, components which would enhance viral gene expression and DNA replication (Hossain et al., 1997).

It has also been reported that HSV-1 ICP0 protein binds and stabilizes cyclin D3 and colocalizes with it in ND10s (Kawaguchi et al., 1997). However, another group has suggested that HSV-1 prevents the normal increases in levels of cyclin D1 and D3 (Song et al., 2000). HSV-1 was also suggested to induce the accumulation of an intermediately phosphorylated form of pRb and the accumulation of E2F-p107 (p107 is a member of the Rb protein family) and E2F-pRb DNA binding complexes. Decreased E2F-p107 in the cytoplasm, which is normally found in the nucleus, was mediated by ICP4, whereas the nuclear accumulation of E2F-p107 was mediated by ICP8 (Olgiate et al., 1999). Previous

studies had shown that this E2F-p107 activity induced by HSV-1 corresponded to G1/S phase complexes containing cyclin A (Hilton et al., 1995). Furthermore, E2F DNA binding activity normally seen in late G1 and G1/S phase boundary (E2F-p107 and free E2F) was induced by ICP4, ICP27, and replication proteins, such as ICP8, UL30, and UL5 (Hilton et al., 1995). Other studies by the same group have suggested that ICP27 was required for blocking the cell cycle in G1-phase (Song et al., 2001). ICP27 inhibited phosphorylation of pRb, and together with ICP4, ICP0 and *vhs*, it inhibited induction of cyclin D1 and CDK4.

It has also been suggested that levels of cyclin A and B were significantly decreased by 8 hpi, as determined by western blot (Advani et al., 2000a). Another group suggested that CDK2/cyclin A kinase activity was significantly decreased at 8 hpi (Ehmann et al., 2000). Furthermore, this same group showed that expression of cyclin A was completely inhibited at 8 hpi in the presence of serum. However, mock infected cells also failed express cyclin A after 8h in the presence of serum. I have observed that cyclin A levels remained constant for up to 7 hpi (unpublished results).

Immunoprecipitated CDK1 remained active during infection, peaking at 16 hpi (Advani et al., 2000a). In contrast, another group showed that CDK1/cyclin B activity decreased from 16 to 24 hpi and that CDK1 levels were lower in infected cells in comparison to mock-treated cells (Hossain et al., 1997).

Two IE HSV-1 proteins, ICP0 and ICP4, are reported to be phosphorylated by CDKs (Advani et al., 2001), but the identity of the specific CDKs required for HSV-1 replication remains elusive. Rosco preferentially inhibits CDK1, CDK2, CDK5, and CDK7 (Meijer et al., 1997; Wang et al., 2001). Of these CDKs, CDK5 is inactive in

cycling cells, in which PCIs inhibit HSV-1 replication, and CDK1 is not required for the expression of the majority of HSV-1 genes (Advani et al., 2000b). Schang *et al.*, showed that CDK2, cyclin A, and cyclin E (but not CDK1 or CDK7) are expressed in explanted neurons that support HSV-1 replication (Schang et al., 2002b). These results suggest that CDK2 may be important for replication of HSV-1 during reactivation from latency. By extrapolation, CDK2 may also be important during lytic infection. This model is disputed by other groups which have argued that CDK2 activity is inhibited during HSV-1 infection. This proposed CDK2 inhibition was only partial and observed at late times post-infection (8 to 12 hours post-infection) (Ehmann et al., 2000; Advani et al., 2001; Ehmann et al., 2001). In contrast, PCIs inhibit transcription of IE genes in less than 2 hours, E genes in less than 3 hours, and HSV-1 DNA replication in less than 6 hours (Schang et al., 1998; Jordan et al., 1999; Schang et al., 1999; Schang et al., 2000). Considering all these results together, HSV-1 does not appear to clearly modulate the cell cycle. In contrast, HSV-1 replication appears to require CDK activity, in that HSV-1 replication is inhibited in the absence of certain CDK activities.

1.9. Rationale and hypothesis

1.9.1. Hypothesis

The mechanisms whereby ICP4 is recruited into RCs remain unclear. The two most common models for such recruitment suggest that ICP4 may be mediated via its binding sites in the HSV-1 genomes which have accumulated in these compartments (Everett et al., 2004). Alternatively, ICP4 may be recruited by binding to proteins at the

core of the pre-RCs or RCs (Zhong and Hayward, 1997). Furthermore, the role of ICP4 phosphorylation in its recruitment to RCs has not been evaluated.

ICP4 has been shown to be phosphorylated to up to five different states. It had also been previously shown that certain phosphorylations of ICP4 were inhibited by Rosco at 10 hpi (Advani et al., 2001). In the experiments presented in chapter 2, I further discovered that Rosco prevented the localization of ICP4 into RCs. These results suggested then that phosphorylation of ICP4 or a binding partner could be required for the recruitment of ICP4 into RCs. Therefore, the hypothesis tested in this thesis is that ICP4 phosphorylation is required for its recruitment into nuclear replication compartments.

1.9.2. Rationale

The mechanisms whereby ICP4 is recruited into RCs have remained elusive. Understanding the roles that phosphorylation of ICP4 plays in its recruitment into RCs would partially characterize the mechanisms whereby ICP4 is recruited into RCs. This characterization could in turn allow us to further understand how other HSV-1 proteins are recruited to pre-RCs or RCs. It may also allow us to further understand the mechanisms whereby ICP4 homologues in other viruses are recruited to homologous replication compartments. Thus, these studies may have significant relevance for many alpha-herpesviruses.

Understanding the importance of ICP4 phosphorylation on its recruitment into RCs may help us evaluate in the future the identities of the kinases involved. The identification of the kinases would in turn allow us to test its involvement in the

recruitment of the ICP4 homologues in other alpha-herpesviruses, or of other HSV-1 proteins.

1.9.3. Thesis outline

My initial studies were aimed at evaluating whether the effect of Rosco or Flavo on HSV-1 replication was homogeneous in all cells. Alternatively, HSV-1 replication in a small subset of cells might have been resistant to PCIs. I thus analyzed the effects of Rosco and Flavo on ICP4 expression on a cell by cell basis. These studies showed that Rosco or Flavo inhibited ICP4 expression homogeneously in all treated cells. Interestingly, I also observed in these experiments an altered localization of ICP4 in the presence of these drugs. Thus, ICP4 failed to localize to RCs, instead it remained mostly diffused through the nucleus **(Chapter 2)**.

I then decided to continue testing the effects of Rosco on ICP4 recruitment into RCs. In these experiments, Roseo was used as a tool to inhibit proper localization of ICP4. I found that ICP4 phosphorylation was probably required for its recruitment into RCs, and that this recruitment was not an exclusive consequence of the effects of phosphorylation on ICP4 levels, HSV-1 DNA replication, or formation of RCs. Furthermore, I demonstrated that ICP4 binding to its cognate sites is not sufficient for its recruitment into RCs **(Chapter 3)**. I further tested whether binding of ICP4 to other proteins was required for its recruitment. I showed that ICP4 binding to two proteins was not sufficient for its recruitment into RCs either **(Chapter 4)**. Thus, although ICP4 phosphorylation is most likely required for its recruitment into RCs, the mechanisms whereby only phosphorylated ICP4 is recruited still remain unsolved. Further studies

must still be performed to identify and characterize the phosphorylation-dependent mechanisms of ICP4 recruitment into RCs.

1.8. REFERENCES

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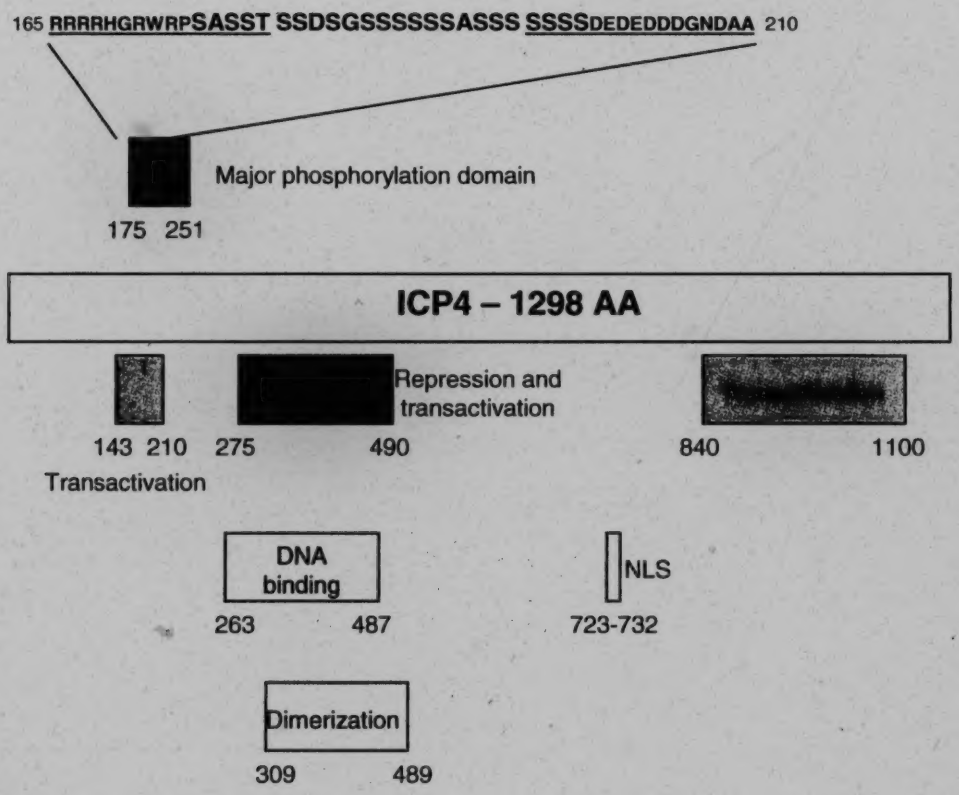

Figure 1.1. Primary structure of ICP4.

Schematic representation of the primary structure of ICP4. The serine-rich domain is highlighted in the sequence shown above in larger and bold font. Consensus phosphorylation sequence for PKA are underlined to the left of the serine-rich tract and for CKII are underlined to the right of the serine-rich tract.

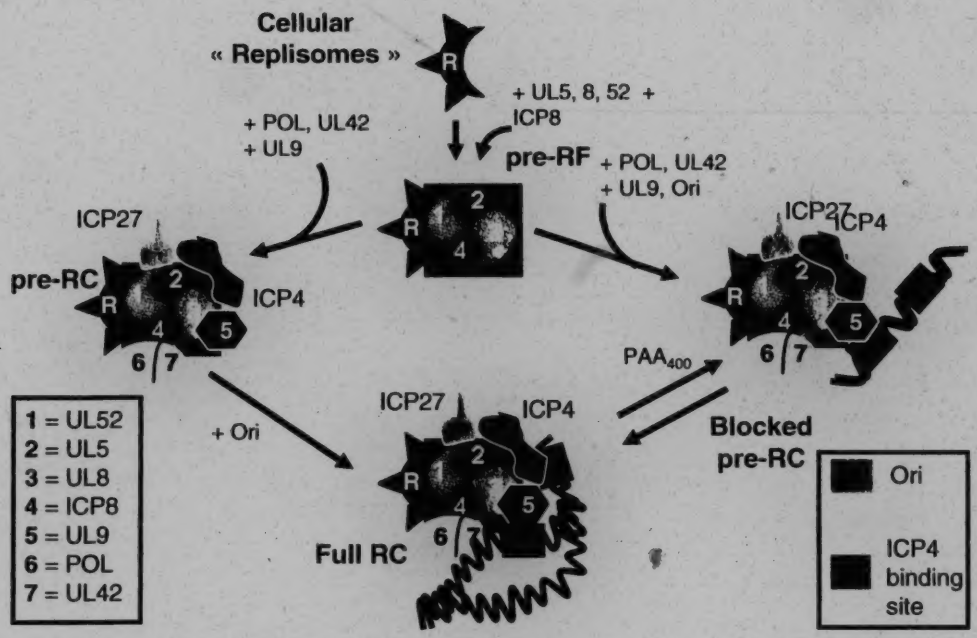

Figure 1.3. A model for the assembly of HSV-1 replication compartments (RCs).
UL5, UL8, UL52, and ICP8 localize to cellular "replisomes" adjacent to ND10s, forming the pre-RFs. UL9, UL42, and UL30 (POL) then localize to the pre-RFs, which upon further accumulation of ICP4 and ICP27 form pre-RCs. Full RCs form after HSV-1 genomes localize to these compartments, allowing HSV-1 transcription and DNA replication. The addition of 400 μg/mL of PAA inhibits the formation of RCs. Model adapted from Zhong and Hayward, 1997.

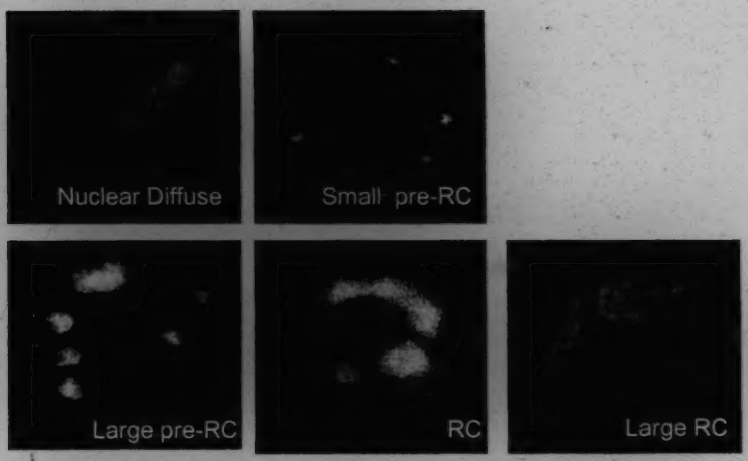

Figure 1.2. ICP4 localization into replication compartments.
Vero cells were infected with 50 PFU of HSV-1 per cell. Cells were fixed and immediate-early protein ICP4 was detected by indirect imunofluorescence.

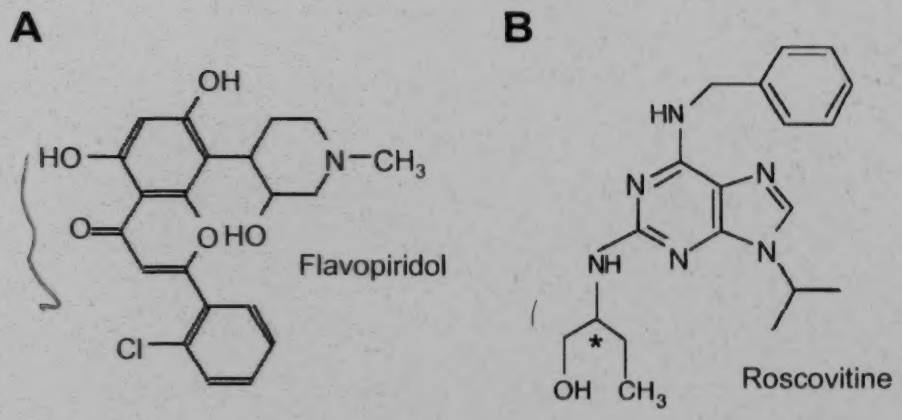

Figure 1.4. Chemical structures of two PCIs.
The chemical structures of flavopiridol **(A)** and roscovitine **(B)** are presented. The asterix in roscovitine indicates the chiral carbon.

CHAPTER 2: PHARMACOLOGICAL CDK INHIBITORS TARGET ICP4 RECRUITMENT INTO REPLICATION COMPARTMENTS AND INITIATION OF VIRAL TRANSCRIPTION

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2.1. INTRODUCTION

Conventional antiviral drugs target viral proteins, thus assuring their specificity and safety. These drugs tend to target only one or a few closely related viruses, however, and quickly select for resistant mutants. Furthermore, viruses with small genomes encode only a few proteins that can be targeted by antiviral drugs, and the proteins of a new pathogen must first be characterized before such drugs can be developed (for recent reviews on cellular and viral proteins as targets for antivirals, see Schang, 2002; Coen and Schaffer, 2003; Provencher et al., 2004; Sadaie et al., 2004; Schang, 2004). In recent years, cellular proteins required for viral replication have started to be considered as potential targets for novel antiviral drugs (for example, see Coen and Schaffer, 2003; de la Fuente et al., 2003; Provencher et al., 2004; Sadaie et al., 2004). Drugs that target cellular proteins required for multiple viral functions may not select for resistant strains as promptly as those that target viral proteins, because mutations in viral genes would have no effect on the targets of the former. Even the viruses with the smallest genomes require a large number of cellular proteins for their replication, thus increasing the number of potential targets for antiviral drugs. And because replication of many unrelated viruses often requires the same

cellular proteins, drugs that target cellular proteins could be used against a novel pathogen even before the proteins encoded by the novel pathogen are characterized.

Most viruses require cellular protein kinases for their replication and a considerable expertise on protein kinase inhibitors has been developed in recent years (recently reviewed in a special issue of BBA 2004). Consequently, cellular protein kinases are attractive targets for novel antiviral drugs. For example, Gleevec, an Abl-tyrosine kinase inhibitor, inhibits poxvirus viral egress. Consequently, poxvirus replication is inhibited. Gleevec also increases survival in infected mice (Reeves et al., 2005). The pharmacological cyclin-dependent kinase (CDK) inhibitors (PCIs) are perhaps the protein kinase inhibitors whose antiviral activities have been most thoroughly characterized. PCIs are a heterogeneous group of compounds that have in common their ability to preferentially inhibit CDKs. Most PCIs are small ($\leq$600 Da), flat, heterocyclic compounds that compete with ATP for binding to the ATP-binding pocket of the target CDKs (recently reviewed in Meijer et al., 1999; Meijer and Damiens, 2000; Fischer and Gianella-Borradori, 2003; Fisher et al., 2003; Schang, 2005). PCIs establish hydrogen bonds with the target CDKs, mostly with main-chain groups or conserved residues in the ATP binding pocket. However, it appears that most of the specificity of PCIs is conferred by their fitting into hydrophobic pockets in the ATP-binding domain of the target CDKs. These pockets are not occupied by the ATP co-substrate and consequently are not so widely conserved among protein kinases.

PCIs can be classified into three groups, non-specific, pan-specific, and oligo-specific (Schang, 2001; Fischer and Gianella-Borradori, 2003). Non-specific PCIs inhibit CDKs and a variety of other protein kinases; pan-specific PCIs inhibit most or all CDKs

indiscriminately; and oligo-specific PCIs have preference for only a subset of CDKs. Mono-specific PCIs may also exist but none has been described. The oligo-specific PCIs can be further classified accordingly to whether they preferentially inhibit CDKs involved in transcription (CDK7, CDK8, and CDK9), or CDKs involved in the regulation of the cell-cycle (CDK1, CDK2, CDK4, CDK6, and CDK7) (Schang, 2001).

PCIs were originally developed as anticancer drugs and are proving to be apparently well tolerated in clinical trials against cancer (for example see Stadler et al., 2000; Shapiro et al., 2001; Benson et al., 2002; Laurence et al., 2002; Benson et al., 2003; Fischer and Gianella-Borradori, 2003; Fisher et al., 2003; Pierga et al., 2003). The dose-limiting toxicity for oligo-specific PCIs are vomiting or diarrhea, which responds to conventional treatments (recently reviewed in Schang, 2002; Provencher et al., 2004; Schang, 2004). Relatively high incidences of thrombosis in patients treated with a pan-specific PCI (flavopiridol) appear to have been caused at least in part by the delivery system used (72 h continuous *iv* infusion) (Schwartz et al., 2001; Shapiro et al., 2001), in that this toxicity was not observed in another Phase II clinical trial in which the same drug was administered as a bolus in daily *iv* applications (Kouroukis et al., 2003).

More recently, PCIs have also been found to inhibit replication of a variety of human pathogenic viruses, including HIV-1, human cytomegalovirus (HCMV), varicella zoster virus (VZV), Epstein-Barr virus (EBV), and herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) (Bresnahan et al., 1996; Schang et al., 1998; Chao et al., 2000; Wang et al., 2001; Kudoh et al., 2004; Schang, 2004; Taylor et al., 2004). Together with their limited serious adverse effects in pre-clinical and clinical trials, the antiviral activities of PCIs make them attractive as potential antivirals. PCIs are currently scheduled to start

Phase I clinical trials as antivirals (www.cyclacel.com). However, the antiviral effectiveness *in vivo* at doses that produce no major adverse effects, their molecular targets, and their antiviral mechanisms must be characterized before PCIs can be developed as clinical antiviral drugs.

Many groups have focused their research efforts in elucidating the antiviral mechanisms of PCIs. These studies have focused mainly on the pan-specific flavonoid flavopiridol (Flavo) and on the oligo-specific purine-type roscovitine (Rosco) (Bresnahan et al., 1997; Flores et al., 1999; Ye et al., 1999; Chao et al., 2000; Schang et al., 2000; Diwan et al., 2004). Flavo inhibits CDKs indiscriminately and Rosco preferentially inhibits CDKs involved in cell-cycle regulation. Different viral functions have been identified as functional targets of PCIs in the different studies. Rosco was shown to prevent initiation of transcription of HSV-1, DNA replication of HCMV and HSV-1, for example, and phosphorylation of structural proteins of VZV (Bresnahan et al., 1997; Ye et al., 1999; Schang et al., 2000; Diwan et al., 2004). In contrast, Flavo was shown to inhibit elongation of HIV transcription (Flores et al., 1999; Chao et al., 2000). It is possible that different PCIs inhibit replication of different viruses through different mechanisms. The studies discussed used different viruses and different PCIs however, which prevent a direct comparison of the different types of PCIs.

In the experiments reported herein, we analyze the antiviral mechanisms of structurally unrelated pan-specific Flavo and the oligo-specific Rosco, which targets preferentially CDKs involved in the cell-cycle against a single model virus, HSV-1. From these studies I conclude that these PCIs prevent initiation of HSV-1 transcription with cell-type dependent potencies. Furthermore, I observed that PCIs also affect the subcellular

localization of HSV-1 proteins. These results further advance our understanding of the antiviral mechanisms of PCIs.

2.2. MATERIALS AND METHODS

2.2.1. Cells and Viruses.

Vero cells (African Green Monkey kidney fibroblasts) and human foreskin fibroblasts (HFF) were maintained in Dulbecco's modified Minimum Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum, 50 mU/ml penicillin, and 50 ng/ml streptomycin (complete medium). A low passage (p10) HSV-1 strain KOS was used throughout this study. Viral stocks were propagated and titrated on monolayers of Vero cells.

2.2.2. HSV-1 infection.

Vero cells were infected with 2.5 or 20 plaque forming units (PFU) of HSV-1 strain KOS per cell in serum free media, as described for each experiment. After 1 h at 37°C, inocula were removed, cells were washed twice with cold phosphate-buffered saline (PBS - 1 mM KH_2PO_4, 154 mM NaCl, 3 mM Na_2HPO_4, pH 7.4), and then incubated in complete media supplemented or not with flavopiridol (Flavo), (*R*)-roscovitine (*R*-Rosco) or (*S*)-roscovitine (*S*-Rosco). HSV-1 replicated with comparable efficiency in HFF or Vero cells in the absence of any drug, with viral yields in the order of 10^7 to $5x10^8$ PFU/10^6 cells in 24 hours (depending on experiment). These yields are in normal ranges, and consistent with our previous experience.

2.2.3. Drugs.

Cycloheximide (CHX) was prepared in serum free DMEM as a 5 mg/ml stock. The stock was diluted to 50 µg/ml in complete medium and added to cells 1 h prior to infection. Flavo, *R*- and *S*-Rosco were a generous gift from Dr. L Meijer (CNRS, Station Biologique Roscoff, Bretagne, France). *R*- and *S*-Rosco were prepared in dimethyl sulfoxide (DMSO) as 100 mM stocks and used at concentrations of 5 to 100 µM. Flavo was diluted in DMSO as a 10 mM stock and used at concentrations of 7.815 to 250 nM. Equivalent amounts of DMSO were added to the medium in the non-drug treated wells. Moreover, these low concentrations of DMSO fail to inhibit HSV-1 replication.

2.2.4. Titrations.

Cells and media were harvested at 24 h post infection, transferred to 14 ml conical tubes and immediately frozen. Samples were further subjected to 3 freeze-thaw cycles and then to 3 cycles of low-energy sonication for 30 s, separated by rest periods of 15 s. Cellular debris was pelleted by centrifugation, supernatants were collected and stored at -80°C. Samples were serially diluted (1:10) and 100 µl of the relevant dilutions were used to infect $1.5x10^5$ Vero cells in 24 well plates, or 3.0×10^5 cells in 12 well plates. Inocula were removed after 1h incubation and 1 ml of 37°C methyl cellulose (2% w/v in complete medium) was added to each well. Infected cells were incubated at 37°C in 5% CO_2 for 3 to 4 days (until plaques were well defined and clear). Cells were fixed and stained with crystal violet in methanol (1% w/v crystal violet, 17% v/v methanol); washed and dried. Individual plaques were then counted.

2.2.4. Toxicity analyses.

Vero or HFF cells were treated as for the viral replication assays, with the exception that no virus was added. After removal of mock-inocula, media containing the different concentrations of drugs were added and the cells were incubated for further 23h as in the viral replication analyses. Cells were then harvested and cell number and viability was analyzed by trypan blue exclusion. Cytostatic effects were defined as either increase in cell numbers in 24 h below that observed in the absence of any drug, or no increase in cell numbers. Cytotoxic effects were defined as increases of more than 3-fold in percentages of non-viable cells over the percentage of non-viable cells in the absence of any drug. Highly cytotoxic effects were defined as increases of more than 6-fold in the percentage of non-viable cells over the percentage of non-viable cells in the absence of any drug. For Vero cells, 2.366% of cells were non-viable in the absence of any drug, and therefore any treatment yielding more than 7.1% of non-viable cells was classified as cytotoxic and any treatment yielding more than 14.2% of non-viable cells was classified as highly cytotoxic. For HFF cells, 9.025% of cells were non-viable in the absence of any drug, and therefore any treatment yielding more than 27.075% of non-viable cells was classified as cytotoxic. No HFF treatment classified as non-cytotoxic yielded more than 14.2% non-viable cells, and no HFF treatment was highly cytotoxic. We did not use MTT or similar mitochondrial toxicity assays because all drugs used are cytostatic at most of the concentrations tested. These assays cannot discriminate between cytostatic or cytotoxic effects.

2.2.5. Run-on analyses.

For each treatment, two 100 mm diameter dishes containing approximately $6x10^5$ Vero cells each were treated for 1h with CHX in complete medium. Cells were then mock-infected or infected with 20 PFU of HSV-1 per cell, in the presence of CHX as indicated. After 1 h adsorption at 37°C, inocula were removed and infected monolayers were washed twice with PBS containing CHX. Cells were then incubated for 4h with complete medium containing CHX. Cells were washed with PBS and then further incubated for 5 h at 37°C with complete medium containing CHX and 100 μM Rosco or 100 nM Flavo. Run-on assays were performed as originally described by Spencer *et al.* (1997) and Rice *et al.* (1995), with several modifications (Diwan et al., 2004). Briefly, cells were resuspended in hypotonic RSB buffer (10 mM Tris pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$), and lysed with 0.5% (v/v) Nonidet P-40. Nuclei were isolated, resuspended in 150 μl Nuclear Freezing Buffer (NFB) (50 mM Tris pH 8.0, 5 mM $MgCl_2$, 40% glycerol, and 0.5 mM dithiothreitol - DTT), immediately snap-frozen, and stored in liquid nitrogen. Afterwards, 150 μl of thawed nuclei suspension was mixed with 150 μl of transcription run-on buffer (20 mM Tris pH 8, 3 mM DTT, 20 mM $MgCl_2$); 0.5mM of each ATP, CTP and UTP; and 10 μCi of [α-^{32}P] GTP. Final buffer concentrations were, 30 mM Tris pH 8.0, 1 mM DTT, 7.5 mM $MgCl_2$, 20% glycerol, 140 mM KCl. Transcription reactions proceeded at 30°C for 30 minutes, and were stopped by incubation with 50 μg (434 Worthington U) of DNase I (Invitrogen, Carlsbad, CA, USA) for 15 minutes at 30°C. Total nuclear RNA was isolated by standard methods. Membranes containing single-stranded DNA were pre-hybridized with 10 ml rapid hybrid buffer (Amersham Biosciences, Piscataway, NJ, USA) at 60°C. Denatured RNA

was added to 5 ml of rapid hybrid buffer at 60°C and hybridized to the membranes for 48 h. Membranes were washed twice for 20 minutes in 300 mM NaCl, 30 mM sodium citrate (2 X SSC), 0.1% SDS at room temperature, and once for 10 minutes in 75 mM NaCl, 7.5 mM sodium citrate (0.5 X SSC), 0.5% SDS at 50°C. Membranes were exposed to Kodak PhosphorImager screens. When indicated, 100 μM Rosco or 100 nM Flavo was added to the run-on transcription reactions.

2.2.6. Immunofluorescence.

For each treatment, approximately $4x10^5$ Vero cells were seeded on coverslips in 24 well plates and infected with 20 PFU of HSV-1 per cell. Inocula were removed following 1 h adsorption at 37°C and the infected monolayers were washed twice with PBS at 4°C. Cells were then treated with complete medium supplemented with 100 nM Flavo, 100 μM *R*-Rosco, 100 μM *S*-Rosco, or vehicle (DMSO). At 5 h post infection, cells were fixed in methanol for 10 min at 4°C. Fixed cells were blocked with 0.5% BSA (bovine serum albumin) in PBS for 30 min. Cells were incubated with primary monoclonal anti-ICP4 antibody (clone 1101 897) diluted 1:500 in 0.5% BSA for 60 min at room temperature, and then washed for 10 min with 0.05% Tween in PBS. Cells were incubated with goat anti-mouse IgG Alexa 594-labeled secondary antibody (Molecular Probes, Eugene, Oregon, USA) diluted 1:500 in 0.5% BSA for 30 min at room temperature, and then washed with 0.05% Tween in PBS for 10 min. Nuclei were counterstained with Hoescht 33258 diluted 1:100 in PBS for 15 min. Coverslips were mounted onto microscope slides, visualized and documented using a fluorescence microscope with a UV light source (Leica DM IRB, Wetzlar, Germany) and camera (QIMAGING RETIGA 1300, Burnaby, Canada). ICP4

signal was quantitated using Quantity One software (BIO-RAD, Mississauga, Canada) and is expressed as arbitrary units.

2.3. RESULTS

2.3.1. The effects of PCIs on HSV-1 replication are cell-type specific.

We first tested the antiviral effects of two structurally unrelated PCIs on the replication of a single virus, HSV-1. Because the antiviral concentrations of Rosco depend on cell-type (Schang et al., 1998), we further compared the PCIs in two cell lines, primary human foreskin fibroblasts (HFF) and immortalized African green monkey kidney cells (Vero). HSV-1 replicates with similar efficiencies in both of these two cell lines, yielding approximately 10^7 to $5x10^8$ PFU/10^6cells in 24 h. Cells were infected with 2.5 PFU of HSV-1 per cell and treated with complete medium supplemented with the indicated concentrations of Flavo, *R*-Rosco, or *S*-Rosco for 24 h **(Figure 2.1)**. To evaluate cell viability, mock-infected cells were incubated with the same concentrations of each drug for 24h. Cell number and viability were then evaluated by trypan blue exclusion.

The antiviral effects of the tested PCIs were cell-type dependent, as expected. Likewise, toxicity was also cell-type dependent. Rosco inhibits HSV-1 replication more potently in fetal human primary lung fibroblasts (HEL) than in immortalized Vero cells (Schang et al., 1998). Consistently, Rosco inhibited HSV-1 replication in neonate HFF cells more potently than in immortalized Vero cells. Albeit to a minor extent, Flavo demonstrated some degree of cell type specificity. Non-cytotoxic concentrations of Flavo inhibited HSV replication by 3.7 orders of magnitude in HFF cells, with an IC_{50} of

20 nM, but by only 3.0 orders of magnitude in Vero cells, with an IC_{50} of 24 nM, **(Figure 2.1 C, D, and Table 2.1)**. Although high concentrations of Flavo apparently inhibited HSV-1 replication to a higher extent in Vero than in HFF cells (by 5.13 and 3.84 orders of magnitude, respectively - **Figure 2.1**), inhibition at these concentrations was actually mediated in part by cytotoxicity (**Figure 2.1**, also Chao and Price, 2001; Lam et al., 2001; Diwan et al., 2004). We have nonetheless included these high concentrations, to compare our results with those of experiments previously reported using them (for example, Chao et al., 2000; Kim et al., 2000; Nelson et al., 2001; Chao et al., 2003). In these experiments, we further compared the antiviral activity of the purified *R*- and *S*-isomers of Rosco. Even though both drugs inhibited HSV-1 replication to approximately the same extent, *S*-Rosco was more potent than *R*-Rosco. Thus, the IC_{50} of *S*-Rosco was 1.5- to 2-fold lower than that of *R*-Rosco in HFF (10 μM and 17.5 μM, respectively) or Vero cells (50 μM and 72 μM, respectively) **(Figure 2.1 A, B, and Table 2.1)**. Some loss of Vero cell viability was observed at 100 μM *R*-Rosco in two of four experiments (average of 9.5% non-viable cells). No cytotoxicity was observed at any concentration of *S*-Rosco.

The results presented in figure 2.1 and table 2.1 confirm that the antiviral effects of PCIs are cell-type specific, as expected for drugs that target cellular proteins. These results also indicated that it was more difficult for any PCI tested to inhibit HSV-1 replication in Vero cells than in HFF cells, which is consistent with previously published experiments (Schang et al., 1998). We used Vero cells for all subsequent experiments on the assumption that any effect that is detectable in Vero cells will likely be even more significant in HFF cells.

2.3.2. PCIs inhibit accumulation of ICP4 into HSV-1 replication compartments.

Our previous results have shown that inhibition of HSV-1 replication by Rosco results from prevention of initiation of HSV-1 transcription (Schang et al., 1999; Diwan et al., 2004) and inhibition of HSV-1 DNA replication (Schang et al., 2000). To evaluate whether the effects on viral gene expression are common to other PCIs or unique to Rosco, I tested the effects of PCIs on HSV-1 gene expression, using the HSV-1 immediate-early protein ICP4 as an indicator. Vero cells were infected with 20 PFU of HSV-1 per cell and treated with vehicle, 100 nM Flavo, 100 μM *R*-Rosco, or 100 μM *S*-Rosco. Although this concentration of Flavo showed some cytotoxic effects at 24 h, I analyzed in these and the following experiments doses of the different drugs that had similar effects on HSV-1 replication. Since these and the following experiments were terminated at 10 h or earlier, no obvious cytotoxicity was observed **(for example, see Figure 2.3)**. Cells were fixed in 4°C methanol at 5 h post infection, ICP4 was detected by immunofluorescence, and ICP4 signal was quantitated using Quantity One software. Quantitation by immunofluorescence was used in these experiments because we wished to compare the levels and accumulation of ICP4 into replication compartments in a cell-by-cell basis **(Chapter 3)**, a comparison that requires quantitation of ICP4 levels in individual cells. We have shown before that PCIs inhibit ICP4 expression as evaluated by metabolic labeling, arguably the most quantitative technique (Schang et al., 1999).

ICP4 was expressed at high levels in the absence of PCIs in two independent experiments, as expected, but at much lower levels in cells infected in the presence of the tested PCIs. As measured by immunofluorescence, ICP4 levels at 5 h post infection were

approximately 2/3 to 3/4 lower in the presence of Flavo, *R*- or *S*-Rosco than in non treated cells **(Figure 2.2)**. Since the levels of ICP4 in non treated cells were saturating for the quantitation system, the degree of inhibition of ICP4 quantitated in these experiments is most likely an underestimate of the actual degree of inhibition. These results indicate that two structurally unrelated PCIs prevent immediate-early viral gene expression to similar extents.

During the analyses of these experiments, I noted that PCIs also appear to affect the subcellular localization of ICP4. HSV-1 genomes and proteins form defined nuclear domains, in which HSV-1 genes are expressed and HSV-1 genomes are replicated. These nuclear domains are referred to as viral replication compartments (Maul et al., 1996). Since ICP4 is among the proteins that localize to the replication compartments, I further examined the formation of these compartments in the presence of PCIs. Replication compartments were identified by the globular accumulation of ICP4 in the nucleus **(Figure 2.3)**. As expected, ICP4 localized to replication compartments in most (approximately 90%) cells infected in the absence of PCIs. In contrast, ICP4 failed to localize to replication compartments in cells infected in the presence of PCIs **(Figure 2.3)**. For example, approximately 1% of cells showed ICP4 accumulation into replication compartments in the presence of Flavo or *S*-Rosco. *R*-Rosco was somewhat less potent than *S*-Rosco and Flavo in these experiments **(Figure 2.3)** leading to 6.8% of cells accumulating ICP4 into replication compartments.

2.3.3. PCIs inhibit HSV-1 transcription.

The results presented in figure 2.2 demonstrate that two PCIs inhibit accumulation of an HSV-1 protein, ICP4, in agreement with previous results which showed that Rosco inhibits transcription of HSV-1 genes (Diwan et al., 2004). Previous results also indicate that Rosco inhibits HSV-1 gene transcription in the presence of immediate-early proteins, such as ICP4 (Schang et al., 1999; Schang, 2004). Therefore, we further evaluated the effects of Flavo on HSV-1 transcription in the presence of these proteins, using a previously described CHX-release experimental design (Schang et al., 1999; Diwan et al., 2004). Vero cells were infected in the presence of CHX with 20 PFU of HSV-1 per cell. After 5 h, cells were transferred to complete medium containing vehicle (DMSO), 100 nM Flavo, or 100 μM Rosco. I have shown that high levels of all HSV-1 immediate-early proteins are synthesized after removal of CHX following this procedure (from the transcripts that over-accumulated during the 5 h incubation in the presence of CHX – see **Chapter 3**). Nuclei were isolated at 10 h post infection, and run-on assays were performed as described (Diwan et al., 2004). We analyzed promoter specific (i.e., sense) and non-promoter specific (i.e., antisense) transcription, by probing with single stranded DNA sense or antisense to selected HSV genes.

Run-on transcription assays performed with nuclei of mock-infected cells (negative controls) resulted in only background levels of hybridization to viral genes. The high background observed for the ICP4 sense probe in CHX is most likely due to cross-hybridization with CHX-inducible cellular RNAs in that it is consistently observed only after CHX treatments. Run-on transcription assays performed with the nuclei of cells infected with HSV-1 in the absence of any drug (positive controls) resulted in abundant transcription of all HSV-1 genes, as expected **(Figure 2.4)**. Most transcription

was from the sense strand (promoter-specific) but a small fraction was from the antisense strand (non-promoter-specific), which is characteristic of HSV-1 transcription (Rice et al., 1995; Spencer et al., 1997; Diwan et al., 2004).

Run-on assays performed with nuclei of cells infected with HSV-1 and maintained in CHX resulted in abundant transcription of the immediate-early gene ICP4 **(Figure 2.4)**. Another IE gene, ICP27, was transcribed under these conditions less efficiently than ICP4, to levels similar to those in the absence of any drug. Since the concentrations of CHX required to allow for an efficient resumption of protein synthesis upon its removal are not sufficient to completely inhibit protein synthesis, some transcription of early and late genes (to lower levels than those in the absence of any drug) are also observed under these conditions. Run-on assays performed with the nuclei of cells infected with HSV-1 in the presence of CHX for 5 h and then further incubated for 5 h with 100 nM Flavo or 100 μM Rosco resulted in almost complete inhibition of transcription of all tested HSV-1 genes **(Figure 2.4)**. PCIs prevented promoter-specific and non-promoter-specific transcription. Flavo, which inhibits transcription elongation, also moderately inhibited transcription when it was present during the run-on transcription **(Figure 2.4)**. Rosco, which acts primarily at or before transcription initiation (Diwan et al., 2004), did not inhibit HSV-1 transcription when present during the run-on transcription **(Figure 2.4)**. These findings are consistent with the lack of effects of Rosco on HSV-1 run-on transcription under similar circumstances (Diwan et al., 2004). Therefore, both Rosco and Flavo cause inhibition of initiation of HSV-1 gene transcription in the presence of immediate-early proteins.

2.4. DISCUSSION

Herein, we show that two structurally unrelated PCIs inhibit viral gene expression. We further show that PCIs affect the subcellular localization of viral proteins and that Rosco and Flavo inhibit initiation of viral transcription, a function that is not targeted by any available antiviral drug. Lastly, we show that *S*-Rosco inhibits HSV-1 replication with IC_{50} approximately 1.5 to 2-fold lower than those of *R*-Rosco.

PCIs inhibit replication of a number of human pathogenic viruses, including HIV-1, HCMV, VZV, EBV, HSV-1 and HSV-2 (Bresnahan et al., 1997; Schang et al., 1998; Chao et al., 2000; Fax et al., 2000; Bhattacharjee et al., 2001; Wang et al., 2001; Schang et al., 2002; Kudoh et al., 2004; Taylor et al., 2004) and are showing only limited serious adverse effects in clinical trials against cancer (Stadler et al., 2000; Shapiro et al., 2001; Benson et al., 2002; Laurence et al., 2002; Benson et al., 2003; Fischer and Gianella-Borradori, 2003; Fisher et al., 2003; Pierga et al., 2003). Consequently, PCIs have been repeatedly proposed as potential antiviral drugs, and Rosco and Flavo have already been tested in an animal model of HIV-induced nephropathy (Bresnahan et al., 1997; Schang et al., 1998; Chao et al., 2000; Meijer, 2000; Coen and Schaffer, 2003; Davido et al., 2003; de la Fuente et al., 2003; Fischer and Gianella-Borradori, 2003; Nelson et al., 2003; Gherardi et al., 2004; Kudoh et al., 2004; Moffat et al., 2004; Sadaie et al., 2004; Schang, 2004). PCIs are now scheduled to enter Phase I clinical trials as antivirals. However, their antiviral mechanisms remain only partially characterized.

One outstanding question is whether different PCIs inhibit viral replication through a common mechanism. Using HSV-1, HCMV, and VZV as models, Rosco has been shown to prevent initiation of viral transcription, DNA replication, and

phosphorylation of viral proteins (Bresnahan et al., 1997; Advani et al., 2001; Diwan et al., 2004; Taylor et al., 2004). Using HIV, Flavo has been shown to inhibit elongation of transcription (Mancebo et al., 1997; Flores et al., 1999; Chao et al., 2000). And using other retroviruses, Rosco and Flavo have also been shown to inhibit expression of cellular proteins required to activate specific viral promoters (Chao et al., 2003). One of the major difficulties in analyzing these results together is that different viruses and cells were used to study the different antiviral effects. Therefore, we tested the effects of two structurally unrelated PCIs with different specificities on a single virus. Flavo preferentially targets CDKs involved in transcription and Rosco that preferentially targets CDKs involved in cell-cycle progression. From these experiments we concluded that PCIs inhibit initiation of viral transcription. However, we have not tested whether PCIs inhibit viral replication only as a result of inhibition of viral transcription. Moreover, we have used only one virus as a model, HSV-1. It is possible, even likely, that PCIs may inhibit different functions of different viruses.

We have previously shown that the antiviral potencies of Rosco are cell-type dependent (Schang et al., 1998). Herein, we show that another PCI, Flavo, also inhibits HSV-1 replication with cell-type dependent potencies **(Figure 2.1)**. Interestingly, the extent of cell-specificity was dependent on the specific drug, in that Flavo was only marginally more potent in HFF than in Vero cells. Although cell-specific potency was expected because PCIs act on cellular targets, the specific mechanisms of these differences are still unknown. Rosco is not substantially metabolized in cultured cells, suggesting that differential metabolism is not likely to account for these differences (Vita et al., 2005). Immortalized or transformed cells could internalize PCIs less efficiently than primary cells,

or export PCIs more efficiently. Transformed cells, for example, commonly overexpress drug-exporting pumps. Over-expression of the multidrug resistance protein 1 (MRP1) confers partial resistance to flavopiridol (Hooijberg et al., 1999), and might indiscriminately confer resistance to other PCIs. However, such genes may or may not be overexpressed in immortalized cells and to the best of our knowledge; their actual levels in Vero cells have not been analyzed. The differences in HSV-1 sensitivity to PCIs in different cells may also reflect the differences in intracellular concentrations of ATP or on levels of other CDK activities. ATP levels and cell-cycle related CDK activities (such as CDK1 and CDK2) are commonly lower in primary than in immortalized or transformed cells. These different levels are consistent with the higher antiviral potencies of PCIs in primary than in immortalized or transformed cells. In contrast, levels of CDK9 or CDK7 activity have not, to the best of our knowledge, been reported to vary among fibroblast cell lines (both HFF and Vero cells are fibroblasts).

High concentrations of Rosco and Flavo could overcome the resistance of Vero cells to their antiviral activities. In the case of Flavo, however, this apparent antiviral activity was mediated, at least in part, by cytotoxicity (**Figure 2.1** and Chao and Price, 2001; Lam et al., 2001; Diwan et al., 2004). High concentrations of Flavo inhibit transcription by RNA polymerase II (Chao and Price, 2001) and, consequently, expression of most cellular genes (Lam et al., 2001). In contrast, the concentrations of Rosco used in these experiments have no major effects on global cellular gene expression, or even on expression of a viral gene recombined in the cellular genome (Lam et al., 2001; Diwan et al., 2004).

PCIs also inhibited localization of ICP4 to viral replication compartments, an effect that has not been described previously. PCIs may directly inhibit the formation of replication compartments by preventing phosphorylation of cellular or viral proteins. This hypothesis is supported by previous reports in which Rosco inhibited phosphorylation of ICP4 (Advani et al., 2001). Rosco was also reported to inhibit phosphorylation or other post-translational modifications of ICP0 (Advani et al., 2001; Davido et al., 2002), another HSV immediate-early protein, which interacts and co-localizes with ICP4. The effects of Rosco on ICP4 phosphorylation, however, have been evaluated only at late times post infection (Advani et al., 2001). Therefore, more experiments are needed before it can be concluded that the effects of PCIs on ICP4 phosphorylation and subnuclear localization are related. Inhibition of ICP4 localization to replication compartments could in turn lead to the previously observed inhibition of HSV-1 DNA replication (Schang et al., 2000). Alternatively, direct inhibition of HSV-1 DNA replication may result in indirect inhibition of the formation of the replication compartments. The experiments presented in chapter 3 were aimed to characterize the mechanisms of inhibition of accumulation of ICP4 into replication compartments.

Since it has one chiral carbon, Rosco has two optical isomers, (*R*) and (*S*). *R*-Rosco inhibited purified CDK1 *in vitro* with an IC_{50} approximately 2-fold lower than that of *S*-Rosco (De Azevedo et al., 1997). *R*-Rosco was also found to co-crystallize preferentially with CDK2, even though both isomers inhibited this kinase with very similar IC_{50} (De Azevedo et al., 1997). In another study, the IC_{50} of *R*- and *S*-Rosco toward CDK2 were dependent on the activating cyclin (cyclin A or cyclin E). Based on these results, it was hypothesized that *R*-Rosco may be biologically the most active

isomer (McClue et al., 2002). Surprisingly, we found that *S*-Rosco inhibits HSV-1 replication with an IC_{50} approximately 1.5- to 2-fold lower than *R*-Rosco (**Figure 2.1** and **Table 2.1**), although both isomers inhibit HSV-1 replication to the same extent at saturating concentrations. According to the results from De Azevedo *et al.* (1997), *R*-Rosco may well be more potent but less specific than *S*-Rosco. Under this scenario, *R*-Rosco would bind to more (irrelevant for HSV-1 replication) cellular proteins than *S*-Rosco. At sub-saturating concentrations, then, more *S*- than *R*- Rosco would be available to inhibit the kinases required for HSV-1 replication. In support of this possibility, it has recently been shown that *R*- and *S*-Rosco show different specificities depending on cell type, species, tissue, and ATP concentrations (Bach et al., 2005). However, the comparative specificities of *R*- and *S*-Rosco in Vero and HFF cells remain to be analyzed.

2.5. CONCLUSION

PCIs have been postulated to inhibit viral gene expression by inhibiting accessibility of transcription factors to viral promoters (Bhattacharjee et al., 2001; Diwan et al., 2004), by inhibiting elongation of viral transcription (Chao et al., 2000), or by inducing down-regulation of expression of cellular genes required for viral transcription (Chao et al., 2003). Our previous results indicate that the effects of Rosco on HSV-1 replication are not mediated by down-regulation of cellular factors, in that Rosco inhibits accumulation of HSV-1 transcripts even when protein synthesis is inhibited by CHX (Diwan et al., 2004). The experiments presented in this chapter, together with previously published results (Diwan et al., 2004), further indicate that Rosco inhibits elongation of run-on HSV-1 transcription inefficiently. Therefore, we conclude that the major antiviral

mechanism of Rosco against HSV-1 is prevention of initiation of viral transcription. This is a novel antiviral mechanism, not used by any current antiviral drug. We have recently shown that this inhibition is genome-specific, but independent of promoter-specific factors (Diwan et al., 2004). Such a mechanism requires no specific viral proteins or sequences and would, therefore, lead to the observed ability of PCIs to inhibit replication of a variety of unrelated viruses. It would also lead to their observed inability to promptly select for drug-resistant strains.

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	HFF		Vero	
PCI	**IC_{50}, μM**	**Maximum inhibition, %***	**IC_{50}, μM**	**Maximum inhibition, %***
Flavo	0.020	99.96	0.024	99.90
R-Rosco	17.5	99.83	72	99.992
S-Rosco	10	99.87	50	99.997

Table 2.1. Comparison of IC_{50} and maximum antiviral effects of *R*-Rosco, *S*-Rosco, and Flavo in two cell lines.

*, Maximum inhibition of HSV-1 replication attained by non-cytotoxic concentrations of the drug, expressed as a percent of viral replication in the absence of any drug for the respective cell types. Experiment was performed by M.D. Urbanowski.

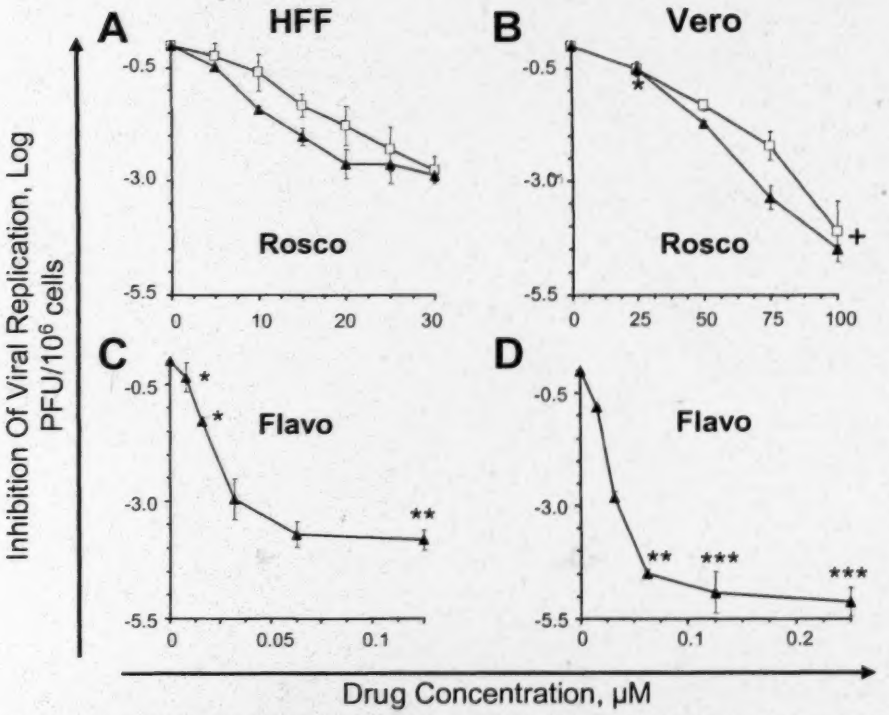

Figure 2.1. HSV-1 replication in Vero and HFF cells in the presence of Flavo, *R*-Rosco or *S*-Rosco.

Inhibition in viral replication per 10^6 cells are plotted as log against drug concentrations. HFF **(HFF)** or Vero cells **(Vero)** were infected with 2.5 PFU of HSV1-KOS per cell. Inocula were removed after 1h, cells were washed twice with 1 ml PBS, and drug solutions in complete medium were added. Cells were treated with either **(A)** 0, 5, 10, 15, 20, 25, or 30 μM *R*- (open squares) or *S*- (black triangles) Rosco, **(B)** 0, 25, 50, 75, 100 μM *R*- (open squares) or *S*- (black triangles) Rosco, **(C)** 0, 7.8125, 15.625, 31.75, 62.5, or 125 nM Flavo, **(D)** 0, 15.625, 31.75, 62.5, 125, or 250 nM Flavo. Virus was harvested at 24 hpi and titrated by standard plaque assays. Note the different scales in the x-axes. As previously reported, antiviral concentrations of these drugs are cytostatic.

*, non cytostatic concentrations; +, probably cytotoxic concentration (percentage of non-viable cells increased ~3 fold above the percentage of non-viable cells in the absence of any drug in two of four experiments); **, cytotoxic concentration (percentage of non-viable cells increased more than three fold above the percentage of non-viable cells in the abcence of any drug); ***, Highly cytotoxic concentration (percentage of nonviable cells increased more than six fold above the percentage of non-viable cells in the absence of any drug. These experiments were performed in collaboration with M.D. Urbanowski, R. Langford, and L.M. Schang.

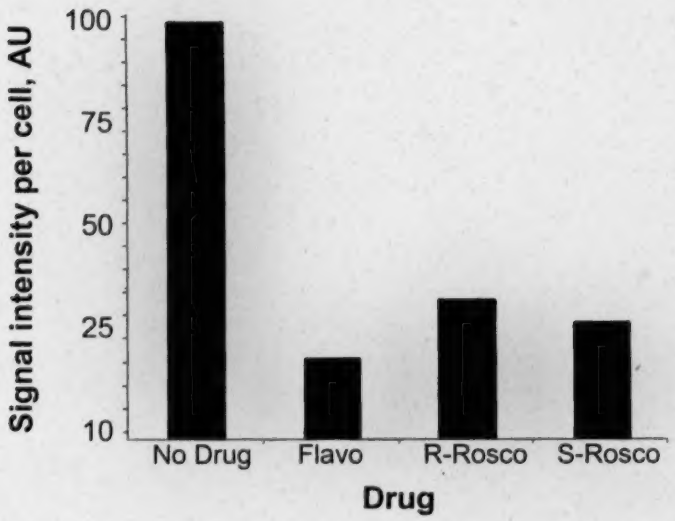

Figure 2.2. Effects of Flavo, *R*-Rosco, or *S*-Rosco on ICP4 expression. Vero cells were infected with 20 PFU of HSV-1 strain KOS per cell. Inocula were removed after 1h, cells were washed twice with cold PBS, and complete medium containing the different drugs was added. Cells were treated with no drug, 100 nM Flavo, 100 μM *R*-Rosco, or 100 μM *S*-Rosco. Cells were fixed at 5 hpi in 4°C methanol. Immediate-early protein ICP4 was visualized by immunofluorescence with anti-ICP4 primary antibody and Alexa 594 conjugated secondary antibody. ICP4 signal was quantitated using Quantity One software and are plotted as arbitrary units (AU). X-axis is at the background level. The results presented are from one of two qualitatively identical experiments (the absolute values differed in the two experiments).

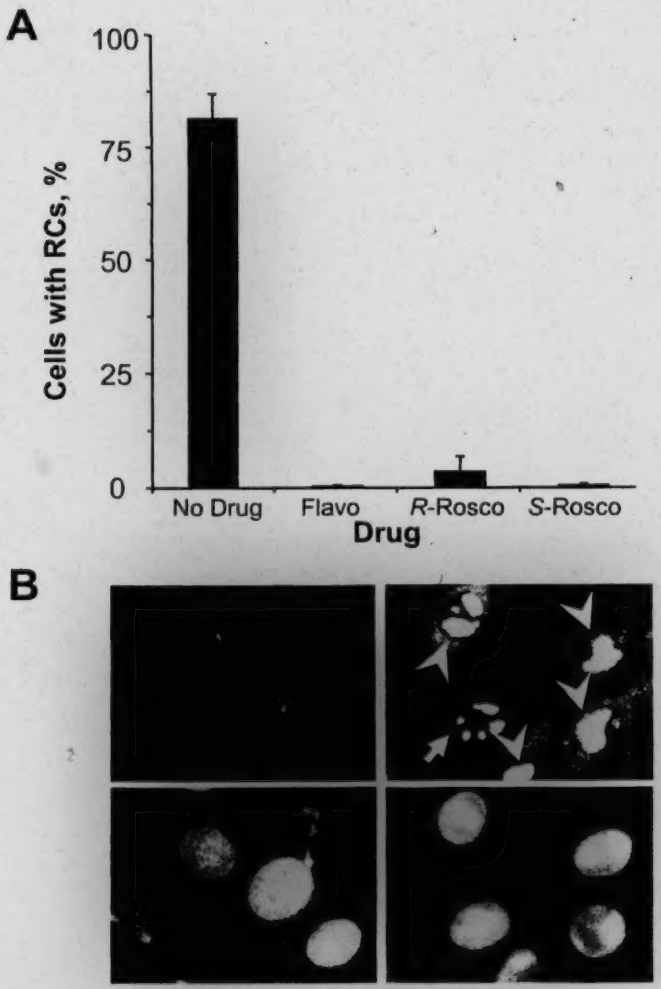

Figure 2.3. Effects of Flavo, R-Rosco, or S-Rosco on accumulation of ICP4 to replication compartments.

(A) Vero cells were infected with 20 PFU of HSV-1 per cell in the presence of no drug, 100 nM Flavo, 100 μM *R*-Rosco, and 100 μM *S*-Rosco and fixed as described in Fig. 2.2. Percentages of cells containing ICP4 accumulated in large pre-replication compartments or replication compartments are plotted against treatment. The results presented are an average of three qualitatively identical experiments. **(B)** Top left panel, representative picture of nuclear diffuse ICP4 in S-Rosco treated infections. Top right panel, representative picture of ICP4 accumulated into large pre-replication compartments or replication compartments (arrows and arrowheads, respectively) in non treated infections. Bottom panels, same fields counterstained with Hoescht 33258.

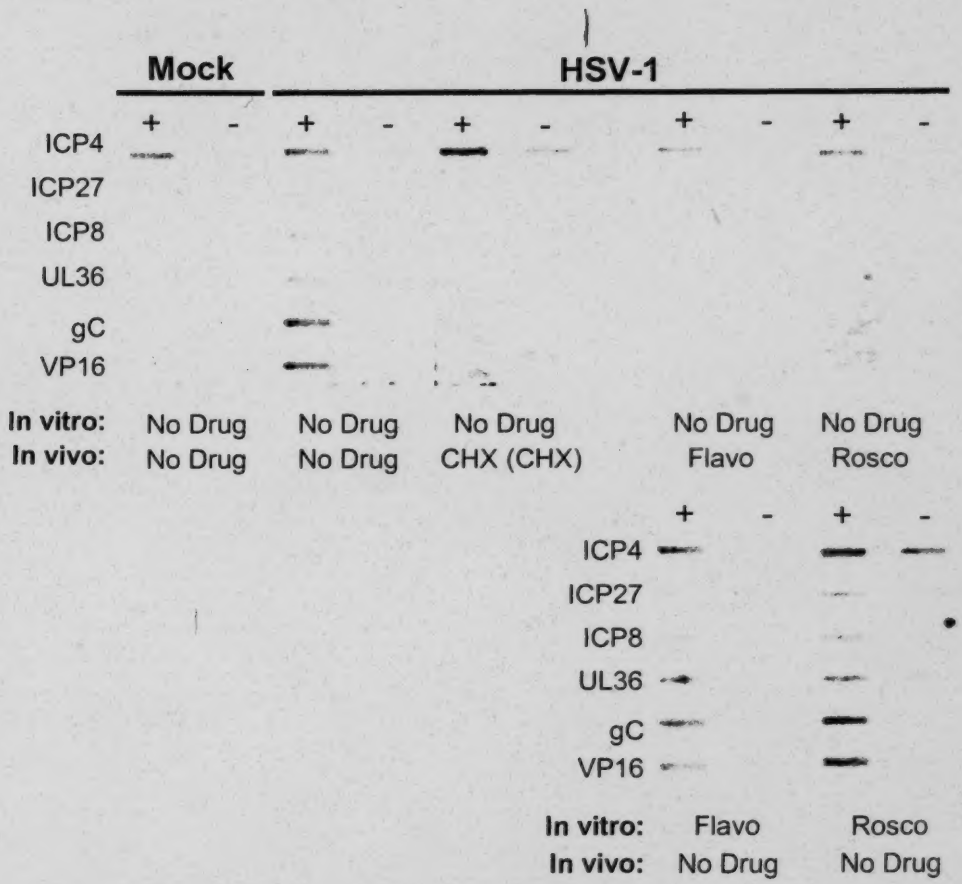

Figure 2.4. PCIs prevet initiation of HSV-1 transcription.

Seven membranes slot-blotted with single-stranded DNA same sense as, or complementary to, six HSV-1 genes and probed with RNA isolated from run-on transcription reactions. Cells were mock infected **(Mock)**, or infected with HSV-1 **(HSV-1)** in the presence of CHX. Five hours later, cells were transferred to medium containing ***(in vivo)*** vehicle **(No Drug)**, CHX **(CHX)**, Flavo **(Flavo)**, or Rosco **(Rosco)**. Nuclei were isolated at 10hpi, and run-on transcription reactions were performed in the presence of ***(in vitro)*** vehicle **(No Drug)**, Flavo **(Flavo)**, or Rosco **(Rosco)**. RNA was purified and probed with membranes containing single-stranded DNA complementary to (+), or same sense as (-), two immediate-early (ICP4, ICP27), two early (ICP8, UL36), and two late (gC, VP16) HSV-1 genes. The higher background in the ICP4 sense probe is consistently observed after CHX-treatments, and is most likely due to cross-hybridization with CHX-inducible cellular RNAs. This experiment was performed in collaboration with J.J. Lacasse.

CHAPTER 3: ICP4 PHOSPHORYLATION IS REQUIRED FOR ITS RECRUITMENT INTO NUCLEAR REPLICATION COMPARTMENTS

A version of this chapter will be submitted for publication. Provencher, V.M.I. and L.M. Schang.

3.1. INTRODUCTION

Herpes simplex virus type-1 (HSV-1) is a large, enveloped, dsDNA virus that replicates in the nucleus. The HSV-1 genome encodes approximately 100 proteins, which are classified according to kinetics of expression as immediate-early (IE), early (E), and late (L) (Honess and Roizman, 1973; Honess and Roizman, 1974; Kozak and Roizman, 1974; Honess and Roizman, 1975; Clements et al., 1977; Jones and Roizman, 1979; Watson et al., 1979).

Expression of HSV-1 proteins is regulated primarily at the transcriptional level (Honess and Roizman, 1974; Wagner, 1985; Godowski and Knipe, 1986; Weinheimer and McKnight, 1987), although important RNA processing and translational control have also been demonstrated (Silverstein and Engelhardt, 1979; Johnson and Spear, 1984; Harris-Hamilton and Bachenheimer, 1985; Su and Knipe, 1989). Transcription of HSV-1 genes is regulated by cis-acting signals in HSV-1 genomes and by viral and cellular trans-acting factors (Zipser et al., 1981; Mackem and Roizman, 1982; Cordingley et al., 1983; Smiley et al., 1983; Whitton et al., 1983; Campbell et al., 1984; Everett, 1984b; Everett, 1984a; Preston et al., 1984; Dynan and Tjian, 1985; Jones and Tjian, 1985; Jones et al., 1985; Coen et al., 1986).

The immediate-early (IE) proteins (ICP0, ICP4, ICP22, ICP27, and ICP47) are transcribed immediately after infection as a result of activation by an HSV-1 virion protein, VP16, acting together with two cellular proteins, HCF and Oct-1 (Post et al., 1981; Batterson and Roizman, 1983; Campbell et al., 1984; Quinlan et al., 1984; O'Hare and Goding, 1988; Preston et al., 1988; Stern et al., 1989; Katan et al., 1990; Xiao and Capone, 1990; Wilson et al., 1993). Four of the IE proteins then regulate further HSV-1 gene expression. Two of them specifically activate transcription of the HSV-1 early (E) genes, ICP4 and ICP0 (Dixon and Schaffer, 1980; Everett, 1984a; DeLuca and Schaffer, 1985; Gelman and Silverstein, 1985; O'Hare and Hayward, 1985; Quinlan and Knipe, 1985; Mavromara-Nazos et al., 1986).

The E genes encode proteins involved in HSV-1 DNA replication, such as the ssDNA binding protein ICP8 (Honess and Roizman, 1973). Expression of E proteins therefore activates HSV-1 DNA replication. The late (L) gene products are expressed following HSV-1 DNA replication, mostly as the proteins required for assembly of progeny virions (Powell et al., 1975; Honess and Watson, 1977; Holland et al., 1980). L gene expression also requires ICP4 and ICP27 (Sacks et al., 1985; Rice and Knipe, 1988; Sekulovich et al., 1988; McCarthy et al., 1989; Su and Knipe, 1989; McMahan and Schaffer, 1990; Rice and Knipe, 1990).

Transcription and replication of HSV-1 genomes occurs in specific nuclear domains, named "replication compartments" (RCs) (de Bruyn Kops and Knipe, 1988; Rice, 1994; Leopardi et al., 1997; Phelan et al., 1997). RCs were originally defined as numerous small punctae of nuclear accumulation of ICP8 (Quinlan et al., 1984), which

was already known to associate with replicating HSV-1 DNA (Powell and Purifoy, 1976; Knipe and Spang, 1982).

RCs are thought to form at pre-existing cellular domains adjacent to ND10s (Zhong and Hayward, 1997). However, the mechanisms whereby RCs are formed are not yet fully understood. In one model, for example, RCs develop from pre-replication foci (pre-RFs). These foci consist of the viral helicase-primase complex (UL5, UL8, and UL52) and the ssDNA binding protein (ICP8) accumulated at pre-existing cellular structures or domains (named "replisomes") (Zhong and Hayward, 1997). Pre-RCs are then formed when the viral polymerase (UL30), the origin binding protein (UL9), and the processivity factor (UL42) are recruited to the pre-RFs (Goodrich et al., 1990; Bush et al., 1991; Calder et al., 1992; Liptak et al., 1996; Lukonis and Weller, 1996). The IE proteins ICP4 and ICP27 also localize to the pre-RFs to form the pre-RCs (Knipe and Smith, 1986; Phelan et al., 1997; Zhong and Hayward, 1997). Full RCs form only when all seven of the HSV-1 DNA replication proteins, ICP4 and ICP27, and the HSV-1 genomes localize to the RCs and HSV-1 replication occurs (Knipe and Smith, 1986; Knipe et al., 1987; Phelan et al., 1997; Uprichard and Knipe, 1997; Zhong and Hayward, 1997). The full RCs are hypothesized to form by coalescence of stationary growing small pre-RCs or by movement of small pre-RCs which merge with adjacent compartments (Taylor et al., 2003).

Under the discussed model, ICP8, UL5, UL8, UL9, and UL52 would form the scaffold required for the structure of the RCs. UL30 would then be recruited in a process that requires the presence of the active primase subunit (UL52) and thus primer synthesis.

UL42 would be the last protein to be recruited to the pre-RCs (Carrington-Lawrence and Weller, 2003).

The mechanisms of recruitment of different proteins are understood to different extents. For example, the C-terminal alpha-helix domain of ICP8 is critical for targeting to RCs. This domain interacts with viral or cellular factors that target ICP8 and its associated proteins to pre-RCs (Taylor and Knipe, 2003). ICP8 has been hypothesized to undergo a conformational change either before, during, or as a result of its localization to pre-RCs and RCs (Uprichard and Knipe, 2003). These changes in ICP8 would then regulate its interactions with the HSV-1 helicase-primase complex and its ability to bind DNA (Uprichard and Knipe, 2003). In contrast, the mechanisms whereby ICP4 is recruited into RCs remain mostly unknown. ICP4 may be recruited by the ICP4 binding sites in the HSV-1 genomes accumulated in the RCs (Everett et al., 2004), or it may be recruited by binding to proteins at the core of the RCs (Zhong and Hayward, 1997).

The nuclear 175 kilodalton (kDa) IE ICP4 protein exists in several phosphorylated forms. Three phosphorylated forms of ICP4 are resolved by one-dimensional SDS-PAGE analyses (Courtney and Benyesh-Melnick, 1974; Pereira et al., 1977). One form is stable, whereas two others rapidly cycle (Wilcox et al., 1980). Finer two-dimensional gel electrophoresis has further identified two additional differentially phosphorylated forms (Advani et al., 2001).

Several protein kinases have been suggested to phosphorylate ICP4, including casein kinase II (CKII), cAMP-dependent protein kinase A (PKA), protein kinase C (PKC), and cyclin-dependent kinase 1 (CDK1) (Xia et al., 1996a; Xia et al., 1996b; Advani et al., 2001). The majority of the phosphorylation sites on ICP4 are located

within a serine rich region in the N-terminal domain (Xia et al., 1996a; Xia et al., 1996b). Phosphorylation of the serine-rich N-terminal domain has been ascribed to PKA, PKC, and CKII (Xia et al., 1996a; Xia et al., 1996b). The serine-rich domain may also stimulate phosphorylation of other domains of ICP4, possibly by regulating the global conformation of ICP4. It has also been postulated that multiple sites on ICP4 may be phosphorylated sequentially and processively (Xia et al., 1996a). Deletion of the serine-rich domain leads to a decrease in ICP4 transactivation of E and L genes (Xia et al., 1996a; Xia et al., 1996b), whereas the effects of these deletions on subnuclear localization have been not analyzed.

As suggested by Papavassiliou *et al.*, phosphorylation of ICP4 most likely occurs on the surface that interacts with other proteins, such as the general transcription factors (TFs) (Papavassiliou et al., 1991). Therefore, phosphorylation would not be expected to regulate DNA binding. Indeed, phosphorylation of ICP4 does not affect high-affinity binding to its ATCGTC cognate sites, which are found in the IE promoters. These cognate sites are responsible for inhibition of IE gene transcription by ICP4. In contrast, no specific ICP4 binding sites have been identified on E and L gene promoters that is responsible for their activation (Everett, 1984a; Imbalzano et al., 1990; Smiley et al., 1992).

Our previous results have shown that Rosco prevented localization of ICP4 to RCs (Lacasse et al., 2005). Based on these results, my hypothesis is that ICP4 phosphorylation is required for its recruitment into RCs. However, the effect of Rosco on ICP4 recruitment could have been secondary to its inhibition of HSV-1 DNA replication. It could also have been secondary to inhibition of formation of RCs, or to the low levels

of ICP4 expressed in the presence of Rosco. Herein, I show that phosphorylation of ICP4 is likely required for its recruitment into RCs, in that inhibition of ICP4 phosphorylation by Rosco correlated with inhibition of its recruitment into RCs. This inhibition was not an exclusive consequence of inhibition of HSV-1 DNA replication, formation of RCs, or ICP4 expression levels. I further show that binding of ICP4 to its cognate sites is not sufficient for recruitment into RCs.

3.2. MATERIALS AND METHODS

3.2.1. Cells and viruses.

Vero cells (African Green Monkey kidney fibroblasts) were maintained in Dulbecco's modified Minimum Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum, 50 mU/ml penicillin and 50 ng/ml streptomycin. Low passage (p10) herpes simplex virus type-1 (HSV-1), strain KOS, was used throughout this study. Viral stocks were prepared and titrated on monolayers of Vero cells.

3.2.2. HSV-1 infection.

5×10^5 Vero cells were infected with 50 plaque forming units (PFU) of HSV-1 per cell in serum-free media, unless otherwise indicated. Inocula were removed after 1h at 37°C and cells were washed twice with cold phosphate-buffered saline (PBS – 1 mM KH_2PO_4, 154 mM NaCl, 3 mM Na_2HPO_4, pH 7.4). Cells were then incubated in complete medium supplemented with the indicated concentrations of cycloheximide (CHX), phosphonoacetic acid (PAA), or roscovitine (Rosco).

3.2.3. Drugs.

CHX was purchased from Sigma (Oakville, Ontario, Canada), prepared in serum-free DMEM as a 5 mg/ml stock and stored at 4ºC. The stock was diluted to 50 μg/ml in complete medium and added to cells 1h prior to infection. Purified *R*- and *S*-isomers of Rosco were a generous gift from Dr. L. Meijer (CNRS, Station Biologique Roscoff, France). *R*- and *S*-Rosco were prepared in DMSO as 100 mM stocks, stored at -20ºC and used at the indicated concentrations. Equivalent amounts of DMSO were added to the medium in the non-drug treated wells. The racemic Rosco mixture was purchased from LC laboratories (Woburn, MA, USA), prepared in DMSO as 100 mM stock, stored at -20ºC, and used at concentrations of 37 or 100 μM. Phosphonoacetic acid (PAA) was purchased from Sigma (Oakville, Ontario, Canada). PAA was prepared in serum-free DMEM as a 100 mg/ml stock, stored at -20ºC, and used at concentrations of 50 or 400 μg/ml.

3.2.4. Immunofluorescence.

5×10^5 Vero cells were seeded on coverslips in 24 well plates the night prior to infection. Cells were infected and then fixed for 10 min in methanol at 4ºC at the indicated times. Fixed cells were blocked with 0.5% bovine serum albumin (BSA) in PBS for 30 min. Blocked cells were incubated for 60 min at room temperature with primary monoclonal anti-ICP4 antibody (clone 1101 897 – Rambaugh-Goodwin Institute for Cancer Research, Inc., Plantation, FL, USA) diluted 1:500 in 0.5% BSA or polyclonal anti-ICP8 antibody (clone 367 – a generous gift from Dr. William Ruyechan, Buffalo, New York, USA) diluted 1:500 in 0.5 % BSA. Cells were then washed for 10 min with

0.05% Tween in PBS. After washing, cells were incubated for 30 min at room temperature with goat anti-mouse immunoglobulin (Ig) G secondary antibody labeled with Alexa 594 (Molecular Probes, Oregon, USA) diluted 1:500 in 0.5% BSA, or with goat anti-rabbit IgG secondary antibody labeled with Texas Red (Vector, California, USA) and diluted 1:100 in 0.5% BSA. Afterwards, cells were washed with 0.05% Tween in PBS for 10 min. Nuclei were then counterstained for 15 min with Hoescht 33258 diluted 1:100 in PBS. Coverslips were mounted onto microscope slides, visualized and documented using a fluorescence microscope with an ultraviolet (UV) light source (Leica DM IRB, Itzlar, Germany) and digital camera (QIMAGING RETIGA 1300, Burnaby, Canada). Between 300-400 cells were counted in approximately 12 fields for each treatment. ICP4 signal intensity per cell was quantitated by selecting single nuclei and subtracting background signal using Quantity One software (BIO-RAD, Mississauga, Canada). ICP4 signal is expressed as arbitrary units.

3.2.5. Hybridization.

Vero cells were seeded in 100mm diameter dishes the night prior to infection and infected with 20 PFU of HSV-1 per cell. Infected cells were treated with complete medium supplemented or not with racemic Rosco (37 μM or 100 μM), PAA (50 μg/ml or 400 μg/ml), or CHX (50 μg/ml). After 6 or 12h of drug treatment, cells were harvested, resuspended in STE buffer (1 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA) and digested with 200 μg/ml proteinase K in 0.5% SDS for 5h at 55°C. DNA was extracted once in phenol:chloroform:isoamyl alcohol (25:24:1) and once in chloroform:isoamyl alcohol (24:1). DNA was then precipitated in the presence of 2.5 volumes of 100%

ethanol at -20°C for 5h, and resuspended in TE pH 7.5 (10 mM Tris-Cl, 1mM EDTA). Samples were run on a 0.6% agarose gel and blotted onto nylon membranes following standard protocols. Membranes were pre-hybridized with rapid hybrid buffer (Amersham Biosciences, NJ, USA) at 80°C, and then hybridized with ^{32}P labeled probe (a mixture of ICP0, ICP4, ICP8, and gC HSV genome fragments, Diwan et al., 2004), in rapid hybrid buffer for 2h at 80°C. Membranes were washed at room temperature twice for 15 min in 300 mM NaCl, 30 mM sodium citrate (2 × SSC), 0.1% SDS. If needed, membranes were then washed once for 15 min in 75 mM NaCl, 7.5 mM sodium citrate (0.5 × SSC), 0.5% SDS at 80°C. Membranes were exposed to Kodak Phosphorimager screens and scanned in a Molecular Imager FX (BIO-RAD, Mississauga, Canada). Signal intensity was quantitated using Quantity One software (BIO-RAD, Mississauga, Canada) and is expressed as arbitrary units.

3.2.6. Analyses of protein phosphorylation in vivo.

Vero cells were seeded in 35mm diameter dishes the night prior to infection and infected in the presence of CHX with 50 PFU per cell of HSV-1. CHX containing media was removed at 6 hpi and cells were treated with 0 or 100 μM Rosco for 4h. Thirty minutes prior to harvest, cells were further treated with 100 μCi $^{32}PO_3^-$ (inorganic orthophosphate). Proteins were then harvested in the presence of phosphatase and protease inhibitors. Samples containing equal radioactivity were run on a 7.5% SDS-PAGE gel and transferred onto nitrocellulose membranes using a semi-dry transfer apparatus. Membranes were exposed to phosphorimager screens and western blotted for ICP0 or ICP4 proteins.

3.2.7. Western blotting.

Membranes were incubated overnight at 4°C in 5% skim milk in Tris Buffered Saline (TBS). Blocked membranes were incubated overnight at 4°C in 5% skim milk with primary monoclonal anti-ICP4 antibody (clone 1101 897 – Rambaugh-Goodwin Institute for Cancer Research, Inc., Plantation, FL, USA) diluted 1:1000 or with monoclonal anti-ICP0 antibody (clone J17) diluted 1:1000. Membranes were then washed 4 times for 5min each with 10% Tween in Tris Buffered Saline (TBST – 50 mM Tris, 150 mM NaCl, and 10% Tween 20, pH 7.5). Membranes were incubated in 5% skim milk for 60 min at room temperature with goat anti-mouse IRDye 800 (Rockland) diluted 1:20,000 or with goat anti-rabbit IRDye 800 (Rockland) diluted in 1:20,000. Afterwards, membranes were washed with TBST 4 times for 5 min each, once with TBS for 5 min, and once with TBS for 30 min. Blots were stored in MilliQ water until scanning in an Odyssey system (LI-COR).

3.2.8. Gel mobility shift assay.

Vero cells were seeded in 100mm diameter dishes the night prior to infection and infected with 50 PFU of HSV-1 strain KOS per cell in the presence of CHX. CHX containing media was removed at 6 hpi and cells were treated with 0 or 100 μM Rosco for 4h. Cells were lysed in lysis buffer (100mM HEPES pH 7.9, 15mM $MgCl_2$, and 100mM KCl). Nuclei were extracted with nuclear extraction buffer (20mM HEPES pH 7.9, 1.5mM $MgCl_2$, 420mM NaCl, 0.2mM EDTA pH 8.0, and 25% (v/v) glycerol) rotated in cold room for 30min and centrifuged at 14000 RPM for 10min. Nuclei were

snap-froze in liquid nitrogen and stored at -80ºC until further use. Oligonucleotides containing the ICP4 DNA core binding sequence CCGAGGACGCCCCGATCGTCCACACCTTC were synthesized at the DNA Core Labs (University of Alberta, Edmonton, Alberta, Canada) (Leopardi et al., 1995). Oligonucleotides were denatured at 100°C for 10min and cooled overnight to room temperature in 500 mL water. Annealed oligonucleotides were then 5'-end labeled with 60 μCi of γ^{32}P-ATP using T4 polynucleotide kinase, following the instructions from the manufacturer (USB Corporation, Cleveland, Ohio, USA). Labeled oligonucleotides were purified by standard ethanol precipitations (once with 100% ethanol and once with 75% ethanol) with 2M ammonium acetate. Labeled oligonucleotides were incubated in a 20 μL binding reaction containing 4% Ficoll, 20mM HEPES pH 7.9, 1mM $MgCl_2$, 0.5mM DTT, 50mM KCl, 8 μg poly dIdC (Amersham Biosciences, Piscataway, New Jersey, USA), 4 μg of nuclear extract, and 0.2 pmol labeled oligonucleotide. Samples were loaded and run on a 5% non-denaturing poly-acrylamide gel electrophoresis (PAGE) for approximately 3h at 120V. The gel was air dried at 30ºC and exposed to phosphoimager screens.

3.2.9. Metabolic labeling and immunoprecipitation assays.

Vero cells were seeded in 35mm diameter dishes the night prior to infection and infected with 50 PFU of HSV-1 per cell in DMEM. At 5 hpi, one dish of infected cells was harvested in RIPA buffer (150mM NaCl, 50mM Tris pH 7.5, 0.1% SDS, 1% NP-40, and 0.5% deoxycholic acid) supplemented with phosphatase and protease inhibitors. Cells in the other dishes were then treated with 0 or 100 μM Rosco in DMEM for 2h.

Thirty minutes prior to harvesting, cells were further treated with 100 μCi/mL $^{32}PO_3^-$ (inorganic orthophosphate). Cells were then harvested in RIPA buffer supplemented with phosphatase and protease inhibitors. Cell lysates of equal counts were precleared with anti-albumin antibody (a generous gift from Dr. Richard Lehner, University of Alberta, Edmonton, Alberta, Canada) diluted 1:1000 in RIPA buffer for 1h at 4°C. Afterwards, 40 μL/mL protein A conjugated agarose slurry (Amersham Biosciences) was added for 1h at 4°C. Precleared supernatants were incubated with monoclonal anti-ICP4 antibody (clone 1101 897 – Rambaugh-Goodwin Institute for Cancer Research, Inc., Plantation, FL, USA) diluted 1:1000 in RIPA buffer for 1h at 4°C. Afterwards, supernatants were further incubated overnight at 4°C with 30 μL/mL protein A conjugated agarose slurry (Amersham Biosciences). Pellets were washed once with RIPA buffer and once with PBS prior to resuspending in 40 μL SDS-PAGE gel loading buffer (200mM Tris-Cl pH 6.8, 8% SDS, 0.2% bromophenol blue, 20% glycerol, and 400mM DTT). Samples were loaded and run on a 7.5% SDS-PAGE gel and gels were air dried overnight at 30°C and exposed to phosphoimager.

3.3. RESULTS

3.3.1. Roscovitine prevents accumulation of the immediate-early protein ICP4 into replication compartments.

Previous experiments from our group have shown that *R*- and *S*-Rosco inhibit expression of ICP4 (Lacasse et al., 2005). These drugs also inhibited accumulation of ICP4 into replication compartments (Lacasse et al., 2005). Rosco was shown by others to inhibit phosphorylation of the most hyperphosphorylated form of ICP4 at late times post-

infection (Advani et al., 2001). Since the effects of ICP4 phosphorylation on its recruitment into RCs remains unknown, I decided to use Rosco as a tool to test such requirements.

To test the effect of phosphorylation on ICP4 accumulation into replication compartments, cells were treated with complete medium containing no drug, 68.75 μM *R*-Rosco (IC_{50} - ***R*-Rosco$_L$**), 100 μM *R*-Rosco (***R*-Rosco$_H$**), 56.25 μM *S*-Rosco (IC_{50} - ***S*-Rosco$_L$**), or 100 μM *S*-Rosco (***S*-Rosco$_H$**). I then evaluated the effect of partial and maximal inhibitory concentrations of Rosco on accumulation of ICP4 into RCs.

Infected cells were fixed at 5 hours post-infection (hpi), and the subnuclear localization of ICP4 in ICP4 expressing cells was evaluated by indirect immunofluorescence. As expected (Schang et al., 1999; Lacasse et al., 2005), IC_{50} or maximal concentrations of *S*- and *R*-Rosco inhibited expression of ICP4 **(Figure 3.1)**. Interestingly, I also observed a differential localization of ICP4 in the presence of *R*- or *S*-Rosco **(Figure 3.1)**. Seventy-two percent of cells infected in the absence of drug had accumulated ICP4 into replication compartments (RCs) at 5hpi. In contrast, no cells had accumulated ICP4 into RCs at 5 hpi in the presence of *R*- or *S*-Rosco.

Since there were no major differences in the effects *R*- or *S*-Rosco on ICP4 accumulation into RCs, I continued these experiments using racemic Rosco. Cells were incubated in complete media supplemented with 37 μM or 100 μM of racemic *R*- and *S*-Rosco (IC_{50} or maximal inhibitory concentration, respectively), or with 50 μg/ml or 400 μg/ml of a HSV-1 DNA polymerase inhibitor, phosphonoacetic acid (PAA) (IC_{50} or maximal inhibitory concentration, respectively). ICP4 expression was then evaluated at 3, 5, 7, and 11 hpi **(Figure 3.2)**.

Almost half (47.8%) of the cells infected in the absence of any drug accumulated ICP4 into small pre-RCs at 3 hpi, whereas the rest (52.2%) showed nuclear diffuse ICP4. ICP4 had accumulated into large pre-RCs in 27.6% of cells at 5 hpi, and into RCs in most cells (99.4%) at 11 hpi. In contrast, 98.25% of cells infected in the presence of 37 μM Rosco had nuclear diffuse ICP4 at 3hpi and the percentage of cells expressing nuclear diffuse ICP4 had only decreased to 58.5% at 7 hpi. The other ICP4-expressing cells accumulated ICP4 mostly into small pre-RCs (33.6%). ICP4 was still nuclear diffuse in 33.45% of cells at 11 hpi, and approximately equal percentages of ICP4-expressing cells had ICP4 accumulated into small pre-RCs (25.6%), large pre-RCs (14.25%), or RCs (26.7%) at this time. When cells were infected in the presence of 100 μM of Rosco, ICP4 never accumulated into RCs during the experiment. Instead, ICP4 remained either nuclear diffusely (66.4%) or within small pre-RCs (24.7%), even at 11 hpi.

As expected, ICP4 did not localize to RCs when cells were infected in the presence of 400 μg/ml PAA. It rather stayed as nuclear diffuse, with a small proportion of cells accumulating ICP4 into small pre-RCs **(Figure 3.2 inset)**. However, ICP4 was localized to similar subnuclear sites as in the absence of any drug when cells were infected in the presence of suboptimal concentrations of PAA (50 μg/ml). At 11 hpi, for example, 84.15% of cells had accumulated ICP4 into RCs, whereas the rest had accumulated it mostly into large pre-RCs (12.85%).

From these experiments I conclude that Rosco prevents ICP4 accumulation into RCs. However, this effect could well be secondary to the low levels of ICP4 expressed under these conditions.

3.3.2. Inhibition of ICP4 accumulation into RCs is not an exclusive consequence of the levels of ICP4.

To evaluate whether ICP4 failed to accumulate into RCs in the presence of Rosco was exclusively a consequence of its low levels of expression, I evaluated the accumulation and levels of ICP4 at the single cell level. Infected cells were incubated with media supplemented with 0 μM, 68.75 μM *R*-Rosco (IC_{50}), 100 μM *R*-Rosco, 56.25 μM *S*-Rosco (IC_{50}), or 100 μM *S*-Rosco, and fixed 5h hours later.

Among the cells infected in the absence of drug, those in which ICP4 was expressed to higher levels tended to display accumulation of ICP4 into higher order compartments. Thus, 82.3% of cells expressing ICP4 levels higher than 100AU had accumulated it into full RCs, whereas 5.9% and 11.8% of these cells showed accumulation of ICP4 into small or large pre-RCs, respectively, and none had nuclear diffuse ICP4. In contrast, 46.1% of the cells expressing ICP4 levels between 75AU and 100AU had accumulated it into large pre-RCs, whereas 15.4% and 38.5% of these cells showed ICP4 accumulated into small pre-RCs, or RCs, respectively, and none had nuclear diffuse ICP4. Among the cells expressing ICP4 levels between 50AU and 75AU, 23.1% had accumulated it into small pre-RCs, 30.8% into large pre-RCs, and 38.5% into RCs, whereas 7.6% of these cells showed nuclear diffuse ICP4. Those cells infected in the absence of drug and expressing low levels of ICP4 had equal possibilities of having ICP4 in either subnuclear distribution. Thus, 27.2% of the cells expressing ICP4 levels lower than 50AU displayed nuclear diffuse ICP4, showed ICP4 accumulated into18.2% into small pre-RCs, and 18.2% into large pre-RCs, whereas 36.4% of these cells

accumulated ICP4 into RCs **(Figure 3.3A)**. Therefore, there is a positive correlation between levels of ICP4 and its localization to RCs in the absence of any drug.

There were not equal differences in ICP4 levels between cells showing different ICP4 subnuclear localizations when the cells were infected in the presence of low or high concentrations of *R*- or *S*-Rosco. In contrast to the cells infected in the absence of drug, no cells infected in the presence of PCIs and expressing ICP4 levels higher than 100AU accumulated ICP4 in RCs. Instead, 28.6% and 71.4% of these cells displayed nuclear diffuse ICP4 or ICP4 accumulated into small pre-RCs, respectively. 72.2% of the cells expressing ICP4 levels between 75AU and 100AU had accumulated it into small pre-RCs, whereas 16.7% and 11.1% and of them displayed nuclear diffuse ICP4 or ICP4 accumulated into large pre-RCs, respectively. Cells expressing ICP4 levels between 50AU and 75AU had equal possibilities of accumulation of ICP4 into small pre-RCs or nuclear diffuse ICP4 (47.9% and 52.1%, respectively). And 70.3% of the cells expressing ICP4 levels lower than 50AU displayed nuclear diffuse ICP4, whereas 29.1% and 0.6% of these cells had accumulated ICP4 into small or large pre-RCs, respectively **(Figure 3.3B)**. Thus, the effects of Rosco on ICP4 recruitment to RCs are not fully accounted for by its effects on ICP4 levels. However, no cells infected in the presence of Rosco had ICP4 levels comparable to those cells showing the highest ICP4 levels among the cells infected in the absence of drug.

3.3.3. Overexpression of ICP4 does not overcome the inhibition of recruitment into RCs.

To test whether ICP4 expressed at high levels could be recruited to RCs in the presence of Rosco, I evaluated next the effects of Rosco on accumulation of overexpressed ICP4. I overexpressed ICP4 using cyclohexamide (CHX) release experimental designs. Cells were infected in the presence of CHX. Following removal of CHX at 6 hpi, infected cells were further incubated with complete media supplemented or not with 100 μM Rosco, 50 μg/ml PAA, or 400 μg/ml PAA. Cells were fixed at 0, 1, 2, and 8 hours after removal of CHX and analyzed by indirect immunofluorescence **(Figure 3.4)**.

One hour after transfer to drug-free medium, 92.15% of cells showed nuclear diffuse ICP4. But ICP4 was recruited into RCs in 86.4% of ICP4 expressing cells at 8h, whereas a small subset of cells still showed ICP4 in small or large pre-RCs **(Figure 3.4)**. One hour after transfer to Rosco-containing medium, ICP4 was also nuclear diffuse in most ICP4 expressing cells (approx. 100%). In contrast to the controls, however, 91.65% of ICP4 positive cells still showed nuclear diffuse ICP4 at 8h post-release. Only a small subset of cells showed ICP4 in small pre-RCs (5.55%).

As expected, ICP4 accumulation displayed a similar pattern after transfer from CHX into 400 μg/ml PAA. Thus, ICP4 was nuclear diffuse in 88.2% of ICP4 expressing cells at 1h post-release and in 94.9% of ICP4 positive cells at 8h **(Figure 3.4 insets)**. The subinhibitory concentration of PAA (50 μg/ml) resulted in ICP4 accumulation reminiscent to that observed in the absence of drug. ICP4 was nuclear diffuse in 98.95% of ICP4 expressing cells at 1h after removal from CHX, but only 8.9% of cells showed nuclear diffuse ICP4 8h after. The majority of ICP4 expressing cells (58.15%) showed

ICP4 accumulation into RCs at this time, whereas 16.05% or 23.05% of cells showed accumulation into small or large pre-RCs, respectively.

I conclude from these experiments that overexpression of ICP4 did not overcome the inhibition of ICP4 accumulation into RCs by Rosco.

3.3.4. Roscovitine inhibits phosphorylation of ICP4 and ICP0.

ICP4 has been previously shown to exist in five differently phosphorylated forms, of which the most hyperphosphorylated was inhibited by Rosco at 10h after infection (Advani et al., 2001). The effects of Rosco on ICP4 phosphorylation at times relevant to the experiments presented in this chapter had not been evaluated. I thus evaluated whether Rosco also inhibited phosphorylation of overexpressed ICP4 at early times after infection. ICP4 transcripts were overexpressed by treating cells with CHX for 6h. Following CHX release, infected cells were incubated with complete media supplemented or not with 100 μM Rosco and ^{32}P. Cells were harvested at 4 hours after removal of CHX in the presence of phosphatase and protease inhibitors. Equal radioactivity of total cell lysates were then resolved on 7.5% SDS-PAGE gels, transferred to nitrocellulose membranes and evaluated by western blotting or autoradiography **(Figure 3.5).**

As expected, ICP4 or ICP0 were not detected immediately after CHX release (time = 0h). Also as expected (Schang et al., 1999), both ICP4 and ICP0 were expressed to high levels 4 hours after the removal from CHX and addition of Rosco. Phosphorylation of both ICP4 and ICP0 was detected at 4 hours in the absence of drug, also as expected **(Figure 3.5A)**, but their phosphorylation was inhibited by Rosco

(Figure 3.5B). Therefore, Rosco inhibited phosphorylation of ICP4. These experiments, however, were not aimed at evaluating whether this inhibition was direct or indirect.

3.3.5. Inhibition of ICP4 phosphorylation by Rosco does not inhibit binding to its cognate sites.

ICP4 binds with high affinity to cognate sites containing the core sequence ATCGTC (Beard et al., 1986; Faber and Wilcox, 1986; Muller, 1987; Kattar-Cooley and Wilcox, 1989; Michael and Roizman, 1989; Wu and Wilcox, 1991). It has been hypothesized that ICP4 is recruited to RCs via its binding to HSV-1 genomes in these sites (Everett et al., 2004). Therefore, Rosco could have inhibited the recruitment of ICP4 into RCs by inhibiting binding of ICP4 to its cognate sites. To test for this possibility, I performed gel mobility shift assays using probes containing ICP4 cognate sites and nuclear extracts from cells infected with HSV-1 in the presence of 0 or 100 μM Rosco.

No shifts in ICP4 mobility were observed in the absence of nuclear extracts or in the presence of nuclear extracts from mock infected cells, as expected since no ICP4 was present in these samples. Also as expected, nuclear extracts from cells infected with HSV-1 in the absence of Rosco induced a shift, which depended on ICP4 in that it was abolished by antibodies against ICP4 **(Figure 3.6)**. Surprisingly, nuclear extracts from cells infected with HSV-1 in the presence of Rosco also induced this shift, which also depended on ICP4 since it was abolished by antibodies against ICP4 **(Figure 3.6)**. From these experiments I conclude that ICP4 binding to its cognate sites is not inhibited by Rosco.

3.3.6. Roscovitine inhibits formation of RCs.

ICP8 is likely the first protein to accumulate to RCs and, therefore, it indicates whether RCs have formed. Rosco could have inhibited recruitment of ICP4 into RCs by inhibiting the formation of these compartments. Thus, I tested next whether Rosco inhibited formation of RCs.

Cells were infected and incubated in complete media supplemented or not with 37 μM Rosco, 100 μM Rosco, 50 μg/mL PAA, or 400 μg/mL PAA. ICP8 expression was then evaluated at 3, 5, 7, and 11 hpi **(Figure 3.7A)**. As expected, 87.3 % of ICP8 expressing cells showed nuclear diffuse ICP8 3h after infection in the absence of any drug. Also as expected, ICP8 had accumulated into RCs in 79.4% of ICP8 expressing cells at 7 hpi, whereas the rest of the cells had accumulated ICP8 within small pre-RCs (13.1%) or large pre-RCs (13.3%). At 11 hpi, ICP8 localized within RCs in all ICP8 expressing cells.

ICP8 expression was delayed in cells infected in the presence of IC_{50} of Rosco, as expected. Moreover, ICP8 was nuclear diffuse in 61.05% of ICP8 expressing cells at 5 hpi, and localized to small pre-RCs in 33.2%. ICP8 eventually accumulated into RCs in 36.75% of ICP8 expressing cells, but only at 11 hpi. ICP8 expression was delayed even further in the presence of 100μM Rosco. Furthermore, ICP8 localized nuclear diffusely in 84.7% of ICP8 expressing cells at 7 hpi and it was still nuclear diffuse in 55.75% of ICP8 expressing cells at 11 hpi. The remaining ICP8-expressing cells had ICP8 localized to small (17.8%) or large (12%) pre-RCs or RCs (4.45%).

As expected, ICP8 did not localize to RCs for 11h in the presence of 400μg/ml PAA. Instead, it localized within small pre-RCs in 72.4% of ICP8 expressing cells and nuclear diffusely in the rest **(Figure 3.7A inset)**. In contrast, ICP8 accumulated into pre-RCs in 23.15% of ICP8 expressing cells, and into RCs in 71.5% at 11 hpi, in the presence of 50 µg/mL PAA.

To further evaluate the formation of RCs in all conditions in which Rosco inhibited recruitment of ICP4, I used the CHX release experimental design previously described. Following removal of CHX, infected cells were further incubated with complete media supplemented or not with 100 µM Rosco, 50 µg/mL PAA, or 400 µg/mL PAA. Cells were fixed at 0, 1, 2, and 8 hours after removal of CHX and analyzed by immunofluorescence **(Figure 3.7B)**.

96.05% of the cells released from CHX into no drug and expressing ICP8 showed nuclear diffuse ICP8 2h later. ICP8 accumulated into RCs in the majority of ICP8 expressing cells at 8h (90.75%), whereas a small subset of ICP8 expressing cells showed ICP8 in small or large pre-RCs at this time **(Figure 3.7B)**. As expected, ICP8 expression was significantly delayed in cells released in the presence of Rosco. Moreover, ICP8 was nuclear diffuse in all ICP8-expressing cells at 8h after removal of CHX.

As expected, ICP8 expression was not delayed in cells released into 400 μg/ml of PAA. Like the untreated cells, 93.4% of ICP8 expressing cells showed nuclear diffuse ICP8 at 2h after removal of CHX. In contrast, only 10.45% of ICP8 expressing cells showed nuclear diffuse ICP8 at 8h, whereas ICP8 localized to small pre-RCs in the vast majority of ICP8 expressing cells **(Figure 3.7B insets)**. Like the untreated cells, 94.35% of ICP8 expressing cells showed nuclear diffuse ICP8 at 2h after release from CHX into

50 μg/ml of PAA. ICP8 localized to RCs in approximately half of the cells at 8h (49.85%), whereas subset of cells showed ICP8 in small or large pre-RCs (16.35% and 32.7% of ICP8-expressing cells, respectively) at this time **(Figure 3.7B)**.

I conclude from these experiments that Rosco prevented accumulation of ICP8 into RCs and thus it likely prevented the formation of these compartments. Therefore, the inhibition of ICP4 accumulation into RCs by Rosco could be exclusively a consequence of its ability to prevent the formation of RCs.

3.3.7. Roscovitine inhibits further phosphorylation of ICP4

To test whether Rosco inhibited phosphorylation of ICP4 in the absence of any pre-treatment with CHX, cells were infected in the absence of any drug for 5h. At 5 hpi, infected cells were further incubated with 0 or 100 μM of Rosco for an additional 2h. 30min prior to harvesting, cells were further incubated with ^{32}P. Equal radioactivity of total cell protein (contained in approximately 100 μg) were immunoprecipitated with ICP4 antibodies, resolved on 7.5% SDS-PAGE, dried, and evaluated by autoradiography.

As expected, ICP4 was phosphorylated at 5hpi and the ICP4 phosphorylation levels had somewhat decreased at 7 hpi (by approximately 23.8%), reflecting that this phosphorylation is dynamic. ICP4 phosphorylation was further inhibited in the presence of Rosco by approximately 66% **(Figure 3.8)**.

I conclude from these experiments that Rosco inhibits phosphorylation of ICP4 in the absence of ICP4 overexpression.

3.3.8. Roscovitine prevents further accumulation of ICP4 into preformed replication compartments.

To evaluate whether the inhibition of ICP4 accumulation into RCs was exclusively secondary to the inhibition of RC formation, infected cells were incubated in complete media for 5h to allow formation of RCs. Infected cells were then fixed or further incubated with complete media supplemented or not with 100 μM Rosco, 50 μg/ml PAA, 400 μg/ml PAA, or 50 μg/ml CHX. Two hours later, cells were fixed and analyzed by immunofluorescence **(Figure 3.9)**.

To evaluate whether Rosco had any effects on further formation of RCs, I analyzed first the localization of ICP8. 75.45% of ICP8-expressing cells showed ICP8 localized to RCs at 5 hpi in the absence of any drug. Two hours later, 18.25% more ICP8-positive cells had accumulated ICP8 into RCs **(Figure 3.9A)**. CHX, 50 μg/mL PAA, or 100 μM Rosco had no major effects on the kinetics of further accumulation of ICP8 into RCs. Thus, 18.3% and 16.1% more ICP8 expressing cells had ICP8 localized to RCs in the presence of CHX or 50 μg/mL PAA, respectively. Rosco did not inhibit ICP8 localization to pre-formed RCs either, in that 21.72% more ICP8 expressing cells accumulated ICP8 into RCs after 2h in 100 μM Rosco.

I concluded from these experiments that Rosco does not inhibit further accumulation of ICP8 into RCs, neither does it disrupt the ICP8 previously accumulated into RCs. I proceeded thus to evaluate the accumulation of ICP4. 49.35% of ICP4 expressing cells had accumulated ICP4 into RCs at 5 hpi in the absence of any drug. Two hours later, 24.55% more ICP4 expressing cells had accumulated ICP4 into RCs, whereas a small subset had accumulated ICP4 in large pre-RCs (5.1%) **(Figure 3.9B)**.

After 2h in 50 μg/ml CHX or 50 μg/ml of PAA, 25.4% or 28% more of the ICP4 expressing cells had accumulated ICP4 into RCs, respectively. After 2h in Rosco, however, only 15.68% more of the ICP4 expressing cells accumulated ICP4 into RCs.

I conclude from these experiments that inhibition of ICP4 accumulation into RCs in the presence of Rosco was not an exclusive consequence of inhibition of RC formation. Furthermore, Rosco inhibited phosphorylation of ICP4 and accumulation of ICP4 into RCs to similar extents (66% and 60.35%, respectively), suggesting that ICP4 phosphorylation is mostly likely required for its recruitment into RCs.

The experiments were then repeated using 400 μg/ml PAA to fully inhibit HSV-1 DNA replication. In these experiments, 39.8% of ICP8 expressing cells had accumulated ICP8 into RCs at 5 hpi. Two hours later, 38.6% more ICP8 expressing cells had accumulated ICP8 into RCs in the absence of drug. In contrast, only 22.6% more ICP8 expressing cells had accumulated ICP8 into RCs in 400 μg/ml of PAA, as expected **(Figure 3.9A insets)**. As expected from these results, 400 μg/ml PAA also inhibited recruitment of ICP4 into RCs. 37.93% of ICP4-expressing cells had accumulated ICP4 into RCs at 5 hpi. As expected, only 15.62% more ICP4-expressing cells had accumulated ICP4 into RCs after 2h in 400 μg/ml of PAA, in comparison to 40.22% more ICP4-expressing cells that had accumulated ICP4 into RCs in the absence of any drug **(Figure 3.9B insets)**.

3.3.9. Inhibition of ICP4 accumulation into RCs by Rosco is not an exclusive consequence of the ability of Rosco to inhibit HSV-1 DNA replication.

Since Rosco inhibits HSV-1 DNA replication (Schang et al., 2000), I further evaluated whether the ability of Rosco to prevent ICP4 accumulation into RCs was due exclusively to its ability to inhibit HSV-1 replication. Cells were incubated in complete media supplemented or not with 37 μM Rosco (IC_{50}), 100 μM Rosco, or 50 μg/ml PAA (IC_{50}). Cells were then harvested 6 or 12h after treatement (7 or 13 hpi, respectively), and DNA replication was evaluated by Southern blot. As previously shown, HSV-1 DNA replication was inhibited in the presence of both concentrations of Rosco **(Figure 3.10A and B).** Furthermore, Rosco inhibited HSV-1 DNA replication to a greater extent than the low concentrations of PAA (50 μg/ml) in these experimental conditions. Consistently with these differences, 50 μg/ml of PAA did not significantly prevent the accumulation of ICP4 into RCs, whereas Rosco did. Thus, the effects on ICP4 recruitment could have been exclusively secondary to the effects on DNA replication.

To further evaluate whether the ability of Rosco to prevent ICP4 accumulation into RCs was due exclusively to its ability to inhibit HSV-1 DNA replication, I tested the effects of Rosco and PAA on HSV-1 DNA replication using the CHX release experimental design previously discussed. Cells were incubated with complete media supplemented or not with 100 µM of Rosco or 50 μg/ml PAA after a CHX release at 6 hpi. Cells were harvested after 6 or 12h of treatment (12 or 24 hpi, respectively) and DNA replication was evaluated by Southern blot. As in the previous experiments, the low concentration of PAA (50 μg/ml) inhibited HSV-1 DNA replication to a lesser extent than 100 µM Rosco **(Figure 3.11A and B).**

I lastly evaluated whether the ability of Rosco to prevent ICP4 accumulation into preformed RCs was due exclusively to its ability to inhibit HSV-1 DNA replication. At 5

hpi, infected cells were incubated with complete media supplemented or not with 100 μM Rosco, 50 μg/ml PAA, or 50 μg/ml CHX. After 6 or 12h of treatment (11 or 17 hpi, respectively) , infected cells were harvested and DNA replication was evaluated by Southern blot. As expected, HSV-1 DNA replication was inhibited in the presence of Rosco **(Figure 3.12A and B).** Under these conditions, however, the low concentrations of PAA (50 μg/ml) inhibited HSV-1 DNA replication as well as, or better than, Rosco. In contrast, such degree of inhibition of HSV-1 DNA replication by 50 μg/ml PAA did not prevent the accumulation of ICP4 into RCs as efficiently as Rosco. Therefore, I conclude from these experiments that inhibition of ICP4 recruitment into RCs by Rosco may not be an exclusive consequence of the effects of Rosco on HSV-1 DNA replication.

3.4. DISCUSSION

Previous studies in our laboratory have shown that Rosco inhibits transcription of immediate-early (IE) and early (E) genes of HSV-1 in the absence or presence of IE proteins and independently of promoter-specific factors (Jordan et al., 1999; Schang et al., 1999; Diwan et al., 2004). I have further shown that both isomers of Rosco, as well as other PCIs, prevent accumulation of ICP4 into RCs (Lacasse et al., 2005). In the experiments described in this chapter, I have further shown now that ICP4 phosphorylation is probably required for its recruitment into RCs. The inhibition of ICP4 recruitment into RCs by Rosco was not an exclusive consequence of the effects of Rosco on ICP4 expression levels, formation of RCs, or HSV-1 DNA replication. Furthermore, ICP4 recruitment to RCs was not an exclusive consequence of binding to its cognate sites in HSV-1 genomes.

The ability of Rosco to disrupt proper localization of viral proteins has been also shown for varicella-zoster virus (VZV). Taylor *et al.,* showed that Rosco prevented normal expression and localization of the VZV ICP4 homologue, IE62 (Taylor et al., 2004). Furthermore, Habran *et al.*, have more recently shown that Rosco also prevented expression and normal localization of VZV IE63 (Habran et al., 2005). My results are fully consistent with these data **(Figures 3.1 and 3.2)**, and I have further shown that overexpression of ICP4 does not overcome inhibition of accumulation into RCs by Rosco **(Figure 3.4).** Therefore, I have shown that the effects of Rosco on proper subnuclear localization of viral proteins into RCs are not entirely a consequence of its effects on expression of these proteins.

Inhibition of ICP4 phosphorylation at late times after infection by Rosco had been previously demonstrated by others. It was proposed that CDK1 phosphorylates ICP4 into its most hyperphosphorylated form, phosphorylation which was inhibited by Rosco (Advani et al., 2001). In these experiments, the Rosco-sensitive hyperphosphorylated ICP4 form was detected only at 10 hpi or later. In addition, I have now shown that Rosco also inhibits phosphorylation of ICP4 as early as 7 hpi (in immunoprecipitation assays) or as early as 4 hours after removal of CHX **(Figure 3.5 and 3.7)**. Since earlier times were not tested, we do not know how soon after infection Rosco inhibits ICP4 phosphorylation. Rosco was also shown to inhibit phosphorylation of VZV IE63 protein (Habran et al., 2005). Furthermore, the authors of these experiments demonstrated that CDK1 and CDK5 phosphorylate IE63 on S224 *in vitro* and that CDK1 phosphorylated this same residue *in vivo.* This phosphorylation was found to be important for proper IE63 localization (Habran et al., 2005).

PCIs inhibited ICP4 localization to RCs in less than 3h **(Figure 3.2)**. Therefore, the kinase activities at late times such as 12 hpi are not very informative with respect to which Rosco-sensitive kinases are required for proper subnuclear localization of IE proteins. In consequence, we still do not know which kinases must phosphorylate ICP4 to direct its recruitment into RCs

I suggest that the five proteins on the autoradiograph shown in figure 3.5 are most likely the five HSV-1 IE proteins. The molecular weights of the observed proteins are consistent with those of the IE proteins on SDS-PAGE gels. Furthermore, the experiments presented in figure 3.5 were performed using the CHX release experimental design. CHX inhibits protein translation, and thus it only allows accumulation of IE transcripts. Thus, Rosco added at the same time when CHX is removed only allows expression of IE proteins (Schang et al., 1999). Therefore, it is unlikely that E and L transcripts would have been translated in sufficient amounts to be detectable in my autoradiograph. Based on this putative identification of the five IE proteins, I further suggest that all five IE proteins likely were phosphorylated in our experiments, as expected since four of the five IE proteins are known phosphoproteins (Wilcox et al., 1980). To the best of my knowledge, however, ICP47 had never been shown to be a phosphoprotein before. Surprisingly, Rosco inhibited phosphorylation of all five putative IE proteins **(Figure 3.5)**, suggesting that the mechanism of inhibition is probably indirect.

Although I hypothesize that ICP4 phosphorylation involves a Rosco-sensitive kinase, other kinases may well be involved in the phosphorylation of ICP4 too. Supporting this model, Rosco inhibited ICP4 phosphorylation only partially, and the levels of phosphorylated ICP4 did not reach background levels when Rosco was added at

5 hpi **(Figure 3.8)**. In contrast, Rosco inhibition of ICP4 phosphorylation was complete when Rosco was added for 4h after CHX removal **(Figure 3.5)**. Thus, some of the ICP4 in the former experiment could have been phosphorylated prior to the addition of Rosco, since Rosco was only added after the formation of RCs. We can not detect this phosphorylation of ICP4 since ^{32}P was only added in the presence of Rosco. There is, however, the possibility that the presence of phosphorylated ICP4 prior to addition of Rosco could induce a conformation of ICP4 that is preferentially phosphorylated at sites that are not affected by Rosco.

cAMP-dependent protein kinase A (PKA) has been suggested to be a major player in the phosphorylation of the serine-rich region of ICP4 (Mullen et al., 1995; Xia et al., 1996a; Xia et al., 1996b). This domain also contains consensus sites for protein kinase C (PKC) and casein kinase II (CKII) (Xia et al., 1996a; Xia et al., 1996b). The serine rich tract consists of a stretch of 35 residues, nineteen serines and one threonine flanked by seven basic amino acids at the N-terminus and eight acidic amino acids at the C-terminus (Xia et al., 1996b). Fifteen of these residues (at positions184-198) are conserved among the ICP4 homologues in HSV-2, VZV, simian varicella herpesvirus, pseudorabies virus, Marek's disease virus, equine herpesvirus type-1, and bovine herpesvirus type-1 (Everett, 1984b; McGeoch et al., 1986; Grundy et al., 1989; Anderson et al., 1992; Schwyzer et al., 1993; Gray et al., 1995). Xia *et al.* suggested that the serine tract is also a target of PKA phosphorylation, and that this phosphorylation is important for the level of HSV-1 replication (Xia et al., 1996b). Deletion of the serine-rich tract (positions 143-210) resulted in reduced viral yields and delayed E and L protein synthesis in tissue culture, and in a block of HSV-1 DNA replication in trigeminal ganglia in mice (Bates and

DeLuca, 1998). The residues that are phosphorylated by PKA have been suggested to interact with transcription factors required for activation of E and L genes (Papavassiliou et al., 1991). In summary, several kinases have been shown to phosphorylate ICP4, and some of them have also been shown to regulate wild-type levels of HSV-1 DNA replication via regulation of ICP4 phosphorylation. However, no protein kinase has yet been shown to be involved in the recruitment of ICP4 into RCs.

The consensus sequence of the ICP4 binding sites is ATCGTC (Faber and Wilcox, 1986). This sequence is conserved among several herpesviruses, as is its binding by the respective ICP4 homologues (Wu and Wilcox, 1991). Although it has been hypothesized that ICP4 recruitment into RCs is mediated via binding to HSV-1 genomes (Everett et al., 2004), the importance of ICP4 DNA binding for its recruitment into RCs (which contain large numbers of HSV-1 genomes) had not been directly tested. I thus tested whether ICP4 binding to its cognate sites was sufficient for recruitment into RCs. To this end, I performed gel mobility shift assays using probes containing ICP4 binding sites and nuclear extracts from HSV-1 infected cells released from CHX in the presence or absence of Rosco. ICP4 binding to its cognate sites was insufficient for recruitment to RCs, in that ICP4 still bound to its cognate sites but was not recruited into RCs in the presence of Rosco **(Figure 3.6)**. DNA binding by ICP4 from cells infected in the presence of Rosco was perhaps expected, since phosphorylated and acid potato phosphatase dephosphorylated ICP4 had previously been shown to bind its cognate sites (Papavassiliou et al., 1991). Furthermore, deletion of residues 143-210 (Shepard et al., 1989) or residues 162-229 (Paterson et al., 1990), which overlap the serine-rich region,

does not affect binding to high affinity binding sites. Furthermore, deletion of residues 143-210 inhibits activation of transcription of E genes (Shepard et al., 1989).

The ability of Rosco to prevent ICP4 accumulation to RCs could have been exclusively secondary to its inhibition of the formation of RCs **(Figures 3.7A and 3.7B)**. However, Rosco had no effect on further accumulation of ICP8 into RCs when the drug was added after RCs had formed **(Figure 3.9A)**. But it did inhibit further accumulation of ICP4 into RCs under the same conditions **(Figure 3.9B)**. Therefore, I conclude that inhibition of ICP4 recruitment to RCs by Rosco is probably not an exclusive consequence of the effects of Rosco on RC formation.

The subnuclear localization of ICP4 and ICP8 is strongly influenced by HSV-1 DNA replication (Cabral et al., 1980; Quinlan et al., 1984). ICP8 localizes to the sites of HSV-1 DNA replication, where it binds single-stranded HSV-1 DNA at the replication bubble (Lee and Knipe, 1983; Puvion-Dutilleul et al., 1985). de Bruyn Kops and Knipe (1988) had suggested that ICP8 is the major organizational protein of RCs, and that it is necessary for recruitment of all other viral and cellular proteins in these compartments. ICP8 normally localizes with a punctuate distribution within RCs, but relocalizes to pre-RCs when DNA synthesis is inhibited (de Bruyn Kops et al., 1998). ICP4 shows a more diffuse distribution within the RCs, and in contrast to ICP8 it remains in this localization when HSV-1 DNA synthesis is inhibited after formation of RCs. In contrast, ICP4 does not localize to pre-RCs when HSV-1 DNA replication is inhibited prior to the formation of pre-RCs (Randall and Dinwoodie, 1986; Knipe et al., 1987). Previous research has shown that Rosco inhibits HSV-1 DNA replication (Schang et al., 2000). Therefore, the effects of Rosco on recruitment of ICP4 into RCs could also have been an exclusive

consequence of its ability to inhibit HSV-1 DNA replication. However, low concentrations of PAA added at 5 hpi resulted in similar inhibition of HSV-1 DNA replication to similar levels as those achieved by Rosco **(Figure 3.12A and B)**, while they did not equally well inhibit the accumulation of ICP4 into RCs **(Figure 3.9B)**. Thus the extent of inhibition of HSV-1 DNA replication by Rosco or 50 μg/ml PAA did not directly correlate with their abilities to inhibit recruitment of ICP4.

High concentrations of PAA added at 5 hpi inhibited HSV-1 DNA replication to a higher extent than Rosco, and also prevented ICP4 accumulation into RCs better than Rosco added at this time **(Figure 3.9B)**. However, these high concentrations of PAA actually prevented the formation of RCs, as evaluated by the accumulation of ICP8 **(Figure 3.9A)**. Therefore, the observed inhibition of ICP4 recruitment to RCs by PAA resulted from its effects on HSV-1 DNA replication and formation of RCs, in contrast to Rosco.

In conclusion, I have shown in this chapter that ICP4 phosphorylation is correlated with its recruitment into RCs. This phosphorylation was inhibited by CDK inhibitors, indicating that it is mediated (directly or indirectly) by Rosco-sensitive kinases.

3.5. REFERENCES

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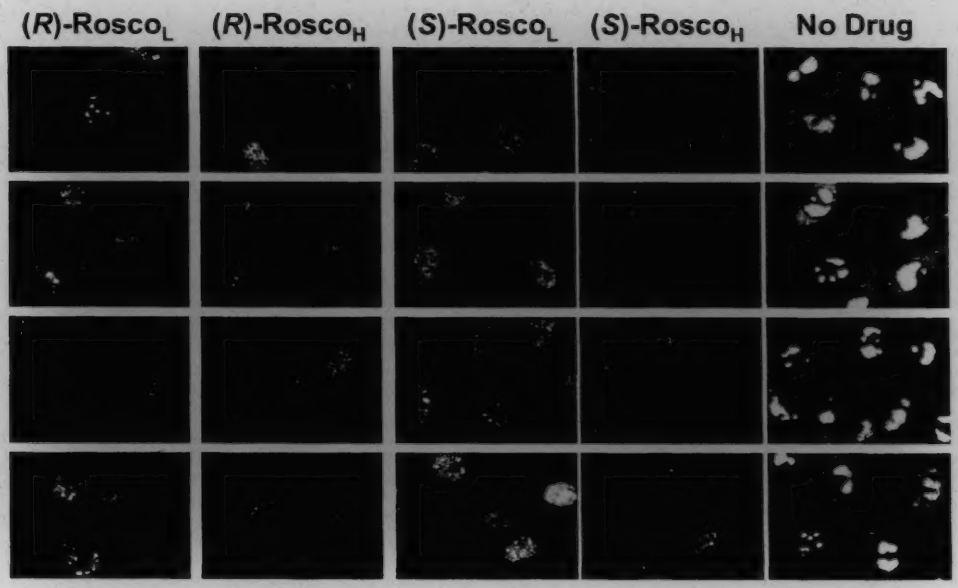

Figure 3.1. Effects of *R*-Rosco or *S*-Rosco on ICP4 expression and its accumulation into replication compartments.

Sixteen representative immunofluorescent pictures of cells infected with HSV-1, treated or not with *R*- or *S*-Rosco, and immunostained for ICP4. Vero cells were infected with 20 PFU of HSV-1 per cell. Infected ells were treated with no drug, 68.75 μM *R*-Rosco (***R*-Rosco$_L$**), 100 μM *R*-Rosco (***R*-Rosco$_H$**), 56.25 μM *S*-Rosco (***S*-Rosco$_L$**), or 100 μM *S*-Rosco (***S*-Rosco$_H$**). Cells were fixed at 5hpi and immediate-early protein ICP4 was detected by immunofluorescence with anti-ICP4 primary antibody and Alexa 594 conjugated secondary antibody. All fields were exposed for the same time. Original magnification 1000A.

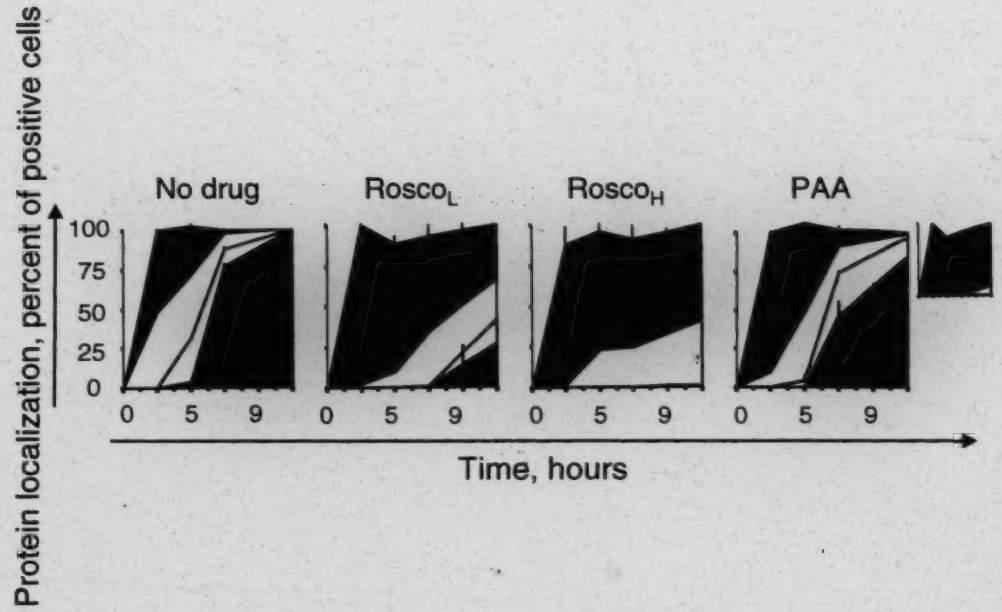

Figure 3.2. Effect of roscovitine on accumulation of ICP4 into replication compartments.

Five area graphs presenting the percentage of ICP4 expressing cells showing nuclear diffuse ICP4 or ICP4 accumulated into pre-RCs or RCs at different times post-infection. Vero cells were infected with 50 PFU of HSV-1 per cell. Inocula were removed after 1h and cells were treated with no drug, 37 μM Rosco (**Rosco$_L$**), 100 μM Rosco (**Rosco$_H$**), 50 μg/ml PAA (**PAA**), or 400 μg/ml PAA (**inset**). At 3, 5, 7, and 11 hpi, cells were fixed and immediate-early protein ICP4 was detected by immunofluorescence. Percentages of ICP4 expressing cells containing ICP4 accumulated nuclear diffuse (**blue**), in small pre-RCs (**yellow**), in large pre-RCs (**green**), or in RCs (**red**) are plotted against time. Error bars represent the range of four independent experiments.

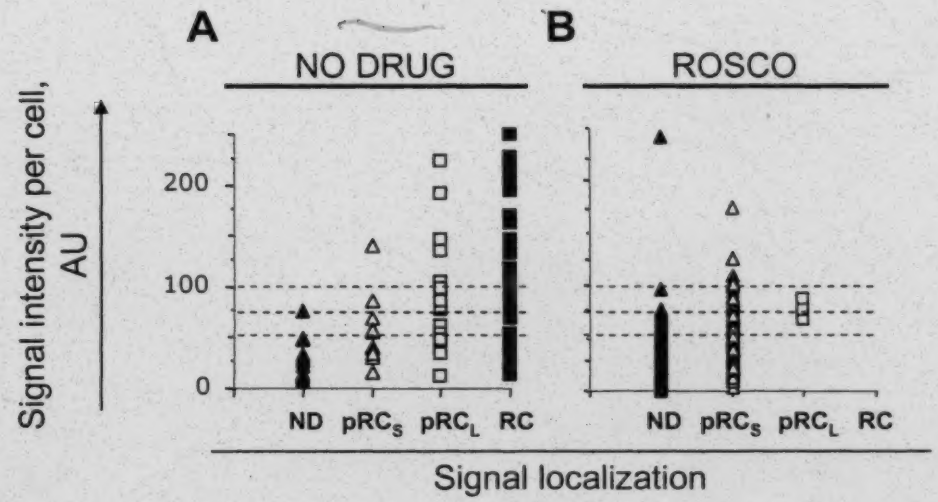

Figure 3.3. Effects of *R*-Rosco or *S*-Rosco on ICP4 levels and subnuclear localization.

Two dot plots presenting ICP4 signal intensity in individual cells plotted against its subnuclear localization. Vero cells were infected with 20 PFU of HSV-1 per cell. After 1h, cells were treated with **(A)** no drug, or **(B)** 72 μM *R*-Rosco, 100 μM *R*-Rosco, 50 μM *S*-Rosco, or 100 μM *S*-Rosco. Cells were fixed at 5 hpi and immediate-early protein ICP4 was detected by immunofluorescence. ICP4 signal was then quantitated in individual cells using Quantity One software and is plotted as arbitrary units (AU) against its subnuclear localization in that given cell. Dashed lines represent signal intensity boundaries to the next higher order compartment (i.e., cells displaying nuclear diffuse ICP4 had ICP4 levels below 50AU).

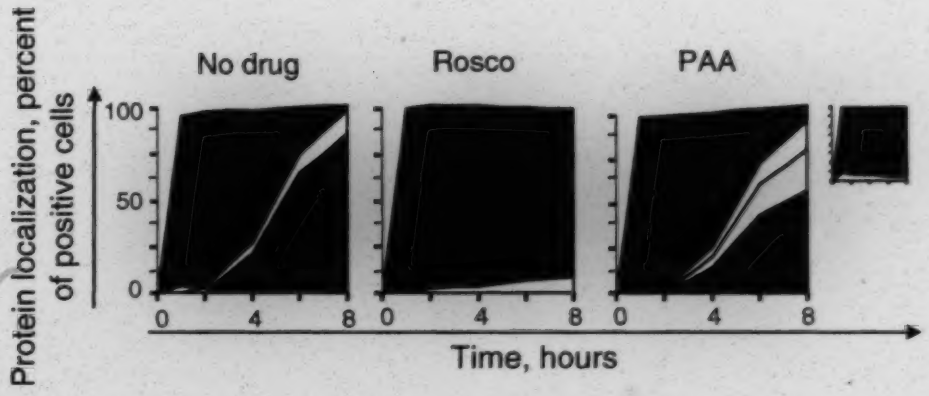

Figure 3.4. Effect of roscovitine on the accumulation of high levels of ICP4 into replication compartments.

Four area graphs presenting percentage of ICP4 expressing cells showing nuclear diffuse ICP4 or ICP4 accumulated into pre-RCs or RCs at different times after release from CHX. Vero cells were infected with 50 PFU of HSV-1 per cell in the presence of CHX. CHX containing media was removed at 6 hpi and cells were treated for 0, 1, 2, and 8h with no drug, 100 μM Rosco, 50 μg/ml PAA, or 400 μg/ml PAA **(inset)**. Cells were then fixed and immediate-early protein ICP4 was detected by immunofluorescence. Percentages of ICP4 expressing cells containing nuclear diffuse ICP4 **(blue)**, and ICP4 accumulated in small pre-RCs **(yellow)**, large pre-RCs **(green)**, or RCs **(red)** are plotted against time after CHX removal. Error bars represent the range of five experiments.

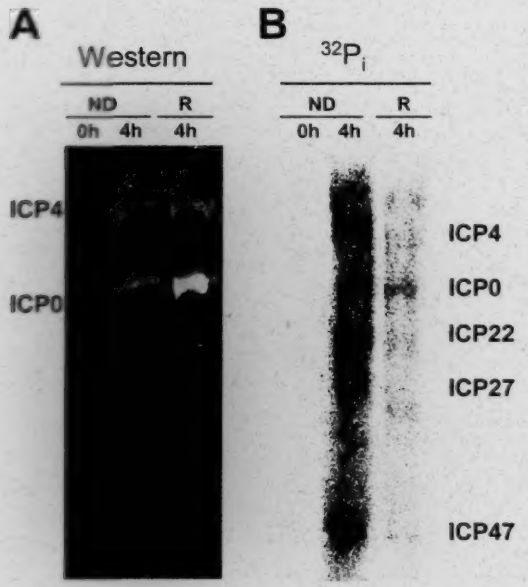

Figure 3.5. Effect of roscovitine on phosphorylation of HSV-1 IE proteins.

A western blot showing expression **(A)** and an autoradiograph showing phosphorylation **(B)** of ICP0, ICP4, and other IE proteins. Vero cells were infected with 50 PFU of HSV-1 per cell in the presence of CHX. CHX containing medium was removed at 6 hpi and cells were treated with no drug **(ND)** or 100 μM Rosco **(R)** for 4h. Thirty minutes prior to harvesting, cells were further treated with 100 μCi $[^{32}P]O_3^-$. Proteins were harvested in the presence of phosphatase and protease inhibitors, resolved in a 7.5% SDS-PAGE gel and either analyzed by western blot for ICP0 and ICP4 **(A)** or by exposure to phosphoimager screens **(B)**.

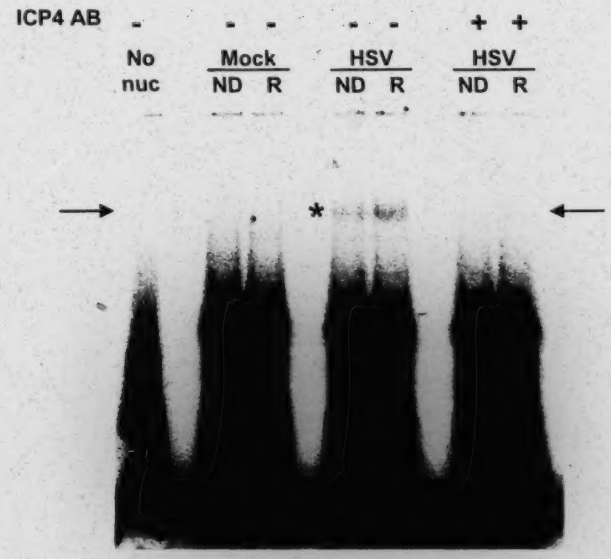

Figure 3.6. Roscovitine does not inhibit ICP4 binding to its cognate sites.

Autoradiograph presenting ICP4-DNA complexes in a gel shift assay. Vero cells were infected with 50 PFU of HSV-1 per cell in the presence of CHX. CHX containing media was removed at 6 hpi and cells were treated for 4h with no drug **(ND)** or 100 μM Rosco **(R)**. Cell nuclei were then harvested. Gel shift assays were performed using a ^{32}P-labeled probe containing the ICP4 binding site core sequence (ATCGTC) in the presence or absence of antibodies specific for ICP4. Samples were resolved on 5% non-denaturing polyacrylamide gel. Gels were air dried and exposed to phosphoimager screens. Arrows represent the ICP4 induced gel shift. The asterix indicates the only samples in which an ICP4-dependent gel shift was observed.

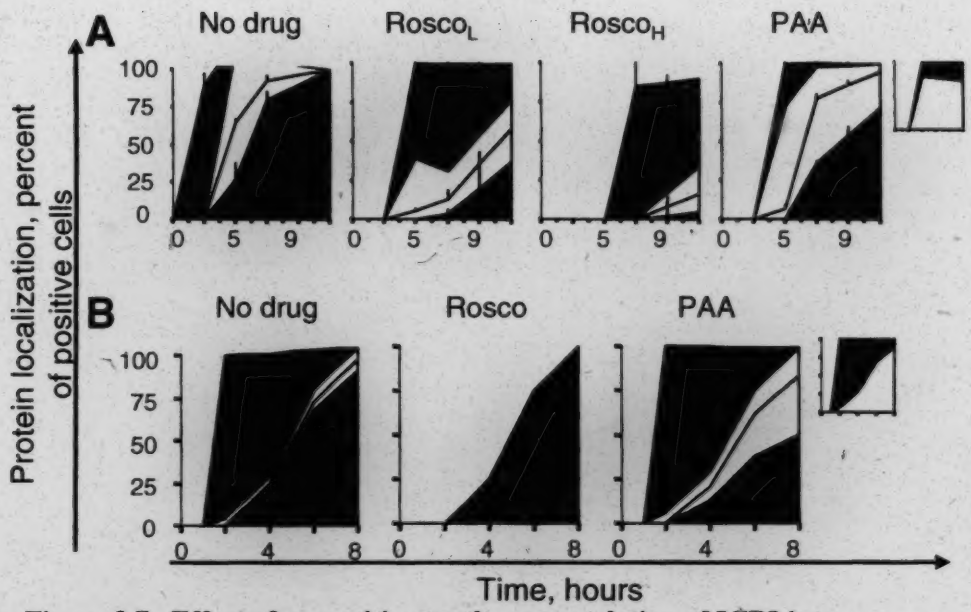

Figure 3.7. Effect of roscovitine on the accumulation of ICP8 into replication compartments.

(A) Five area graphs presenting the percentage of ICP8 expressing cells showing ICP8 nuclear diffuse or ICP8 accumulated into pre-RCs or RCs at different times post-infection. Vero cells were infected with 50 PFU of HSV-1 per cell. Inocula were removed after 1h and cells were treated with no drug, 37 μM Rosco (**Rosco$_L$**), 100 μM Rosco (**Rosco$_H$**), 50 μg/ml PAA (**PAA$_L$**), or 400 μg/ml PAA (**insets**). Cells were fixed at 3, 5, 7, and 11 hpi and early protein ICP8 was detected by immunofluorescence. Percentages of ICP8 expressing cells containing ICP8 accumulated nuclear diffuse (**blue**), in small pre-RCs (**yellow**), in large pre-RCs (**green**), or in RCs (**red**) are plotted against time. Error bars represent the range of four independent experiments.

(B) Four area graphs presenting the percentage of ICP8 expressing cells showing nuclear diffuse ICP8 or ICP8 accumulated into pre-RCs or RCs at the different times post-infection. Vero cells were infected with 50 PFU of HSV-1 per cell in the presence of CHX. At 6 hpi, CHX containing media was removed and cells were treated for 0, 1, 2, and 8h with no drug (**No drug**), 100 μM Rosco (**Rosco**), 50 μg/ml PAA (**PAA**), or 400 μg/ml PAA (**PAA inset**). Cells were then fixed and early protein ICP8 was detected by immunofluorescence. Percentages of ICP8 expressing cells containing nuclear diffuse ICP8 (**blue**), and ICP8 accumulated in small pre-RCs (**yellow**), large pre-RCs (**green**), or RCs (**red**) are plotted against time after CHX removal. Error bars represent the range of five experiments.

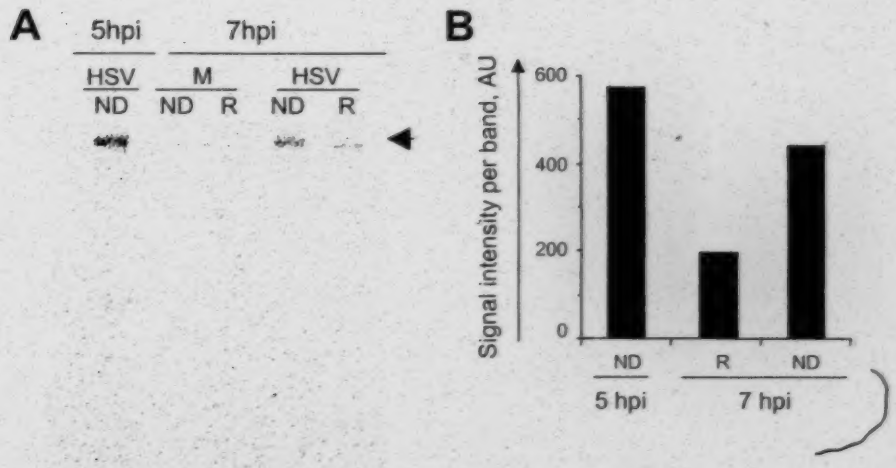

Figure 3.8. Effect of roscovitine on ICP4 phosphorylation.

Autoradiograph showing phosphorylated ICP4 **(A)** and a bar graph presenting the quantitation of ICP4 phosphorylation **(B).** Vero cells were infected with 50 PFU of HSV-1 per cell. Proteins were harvested from one sample at 5 hpi in the presence of protease and phosphatase inhibitors. The other samples were further incubated for 2h in the presence of 0 or 100 μM Rosco. Thirty minutes prior to harvesting, cells were incubated with ^{32}P. Immunoprecipitated proteins were resolved on a 7.5% SDS-PAGE gel, air dried, and evaluated by autoradiography **(A).** Bands were then quantitated using Quantitiy One software. Graph presenting signal intensity, expressed as arbitrary units (AU), plotted against treatment **(B)**.

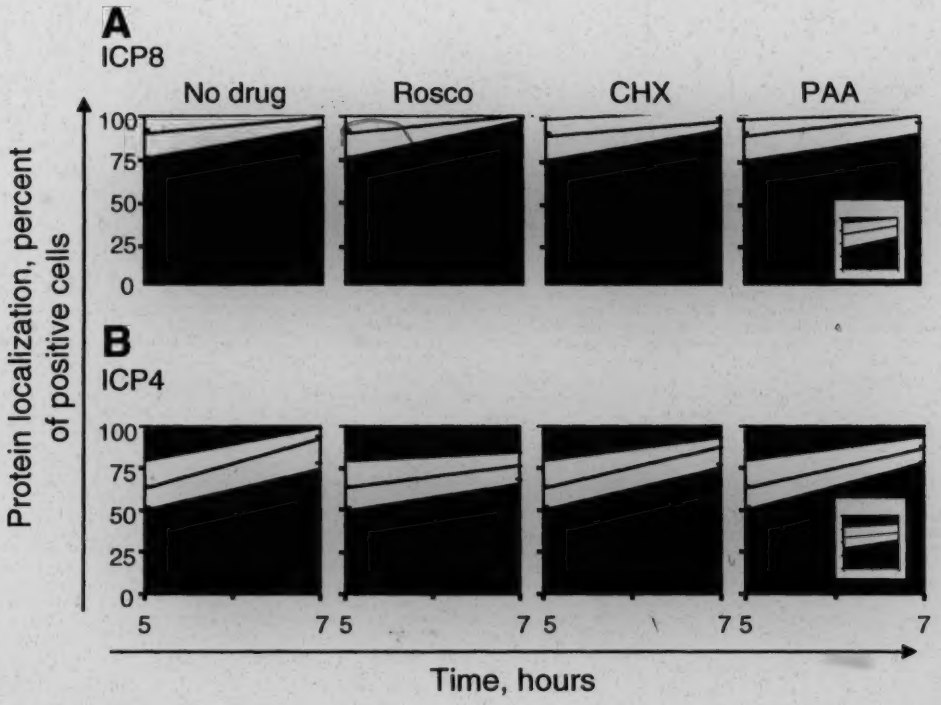

Figure 3.9. Effect of roscovitine on further accumulation of ICP8 and ICP4 into preformed replication compartments.

Ten area graphs presenting the percentage of ICP4 or ICP8-expressing cells showing nuclear diffuse ICP4 or ICP8, or ICP4 or ICP8 accumulated into pre-RCs or RCs at 5 and 7 hpi. Vero cells were infected with 50 PFU of HSV-1 per cell. One set of cells were fixed at 5 hpi. The other sets of cells were treated at 5 hpi for 2h with no drug, 100 μM Rosco, 50 μg/ml PAA, 50 μg/ml CHX, or 400 μg/ml PAA **(insets)**. Cells were then fixed at 7 hpi and early protein ICP8 **(A)** or immediate-early protein ICP4 **(B)** were visualized by imunofluorescence. Percentages of ICP8 **(A)** or ICP4 **(B)** expressing cells containing nuclear diffuse ICP4 or ICP8 **(blue)** , or these proteins accumulated into small pre-RCs **(yellow)**, large pre-RCs **(green)**, or RCs **(red)** plotted against time. Error bars represent the range of four experiments.

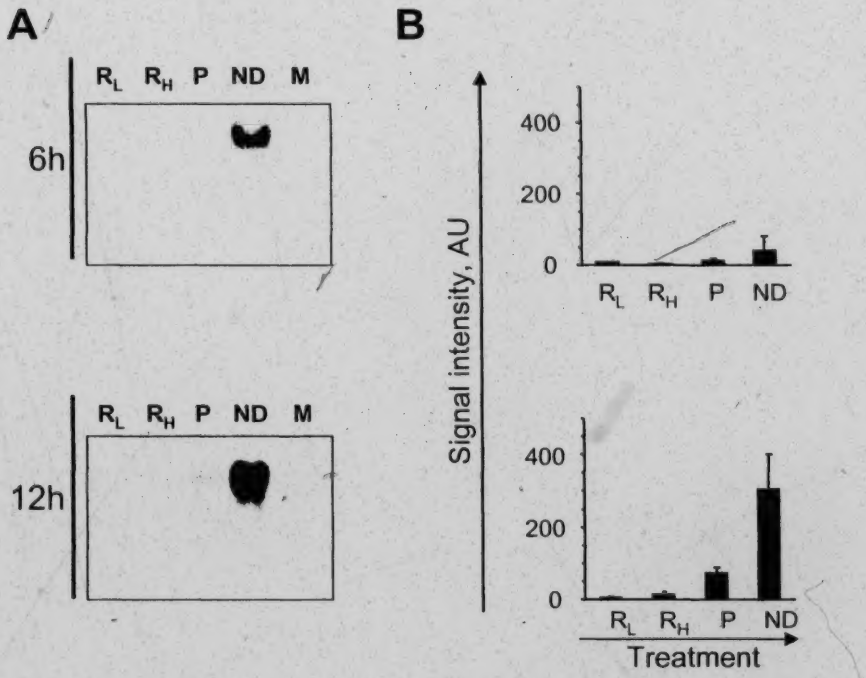

Figure 3.10. Effects of roscovitine on HSV-1 DNA replication.

Two Southern blots from a representative experiment showing HSV-1 DNA levels at 6 and 12 hours post-drug treatment **(A)** and two bars graphs representing the quantitation of HSV-1 DNA levels in two experiments **(B)**. Vero cells were infected with 10 PFU of HSV-1 per cell. At 1 hpi, cells were treated with no drug **(ND)**, 37 μM Rosco **(R_L)**, 100 μM Rosco **(R_H)**, or 50 μg/ml PAA **(P)**. Viral DNA was harvested at 7 and 13 hpi, resolved by agarose gel electrophoresis, blotted, and hybridized with HSV-1 specific probes **(A)**. Two graphs presenting signal intensity expressed as arbitrary units (AU) plotted against drug treatment **(B)**. Error bars represent the range of two independent experiments.

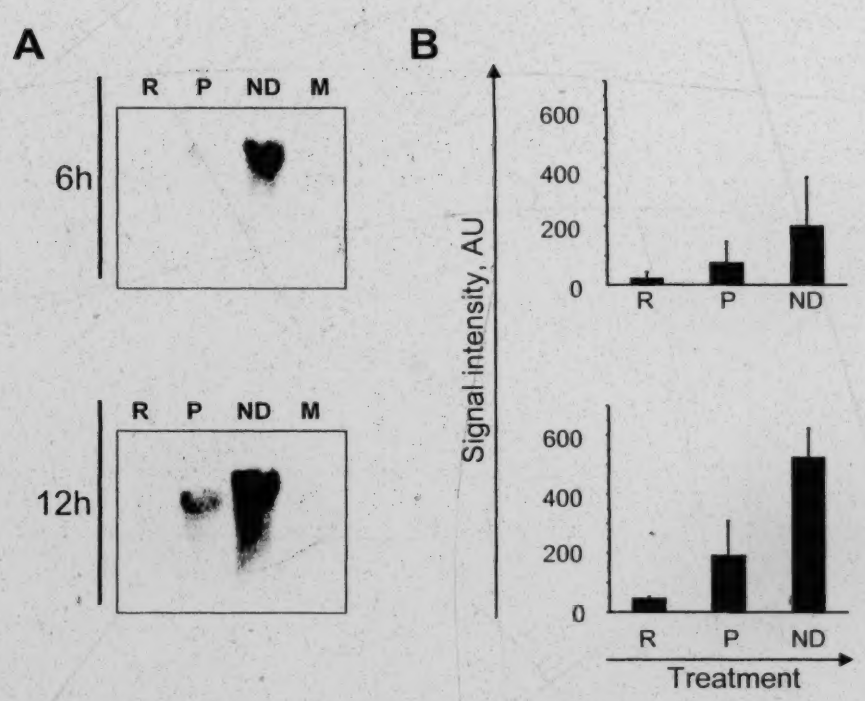

Figure 3.11. Effects of roscovitine on HSV-1 DNA replication following CHX treatment.

Two Southern blots from a representative experiment showing HSV-1 DNA levels at 6 and 12 hours post-drug treatment **(A)** and two bars graphs representing the quantitation of HSV-1 DNA levels in two experiments **(B)**. Vero cells were infected with 10 PFU of HSV-1 per cell in CHX containing media. At 6 hpi, CHX containing media was removed and cells were treated with no drug **(ND)**, 100 μM Rosco **(R)**, or 50 μg/ml PAA **(P)**. Viral DNA was harvested at 12 and 24 hpi, resolved by agarose gel elctrophoresis, blotted, and hybridized with HSV-1 specific probes **(A)**. Two graphs presenting signal intensity expressed as arbitrary units (AU) plotted against drug treatment **(B)**. Error bars represent the range of two independent experiments.

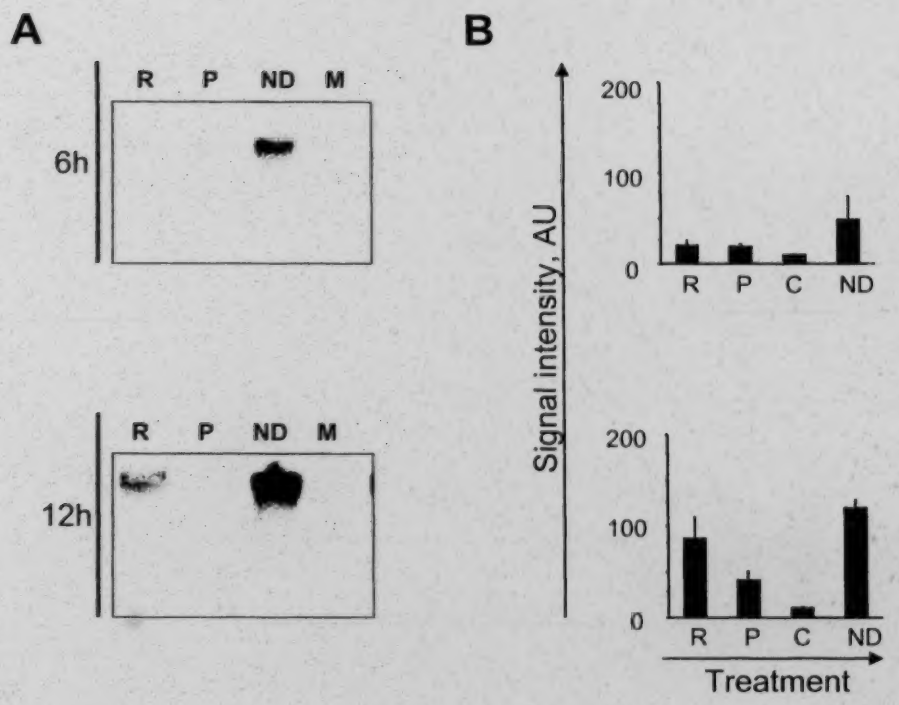

Figure 3.12. Effects of roscovitine on HSV-1 DNA replication after the formation of replication compartments.

Two Southern blots from a representative experiment showing HSV-1 DNA levels at 6 and 12 hours post-drug treatment **(A)** and two bars graphs representing the quantitation of HSV-1 DNA levels in two experiments **(B)**. Vero cells were infected with 10 PFU of HSV-1 per cell. At 5 hpi, cells were treated with no drug **(ND)**, 100 μM Rosco **(R)**, or 50 μg/ml PAA **(P)**. Viral DNA was harvested at 11 and 17 hpj, resolved by agarose gel electrophoresis, blotted, and hybridized with HSV-1 specific probes **(A)**. Two graphs presenting signal intensity expressed as arbitrary units (AU) plotted against drug treatment **(B)**. Error bars represent the range of two independent experiments.

CHAPTER 4: ROSCOVITINE DOES NOT AFFECT ICP4 BINDING TO ITS BINDING PARTNERS

This chapter contains unpublished results.

4.1. INTRODUCTION

In the previous chapters, I have shown that ICP4 phosphorylation is sufficient for its recruitment into replication compartments (RCs). ICP4 recruitment was inhibited when ICP4 phosphorylation was inhibited by roscovitine (Rosco), and this inhibition was not an exclusive consequence of ICP4 expression levels, HSV-1 DNA replication, or RC formation.

ICP4 has been shown to bind ICP4 binding sites containing the ATCGTC core sequence (Beard et al., 1986; Faber and Wilcox, 1986a; Muller, 1987; Kattar-Cooley and Wilcox, 1989; Michael and Roizman, 1989; Wu and Wilcox, 1991). Binding to this sequence at the transcription start site of the IE genes is required to inhibit IE gene transcription. Although the DNA binding region of ICP4 is also essential for activation of transcription of E and L genes (Faber and Wilcox, 1986b; DeLuca and Schaffer, 1988; Michael et al., 1988; Paterson and Everett, 1988; Shepard et al., 1989), no specific ICP4 binding sites have been identified on E and L gene promoters (Everett, 1984; Imbalzano et al., 1990; Smiley et al., 1992). I have shown that ICP4 binding to its cognate sites is not sufficient for its recruitment into RCs either **(Chapter 3)**. As an alternate hypothesis, ICP4 phosphorylation may regulate its binding to specific proteins that recruit it to the RCs, or that sequester it away from them.

In the experiments presented in this chapter, I show that as expected HSV-1 DNA replication was efficiently inhibited by 400 μg/ml PAA in the experiments described in

chapter 3. I also show that ICP4 binding to other proteins does not appear to be sufficient for its recruitment into RCs.

4.2. MATERIALS AND METHODS

4.2.1. Cells and viruses.

Vero cells (African Green Monkey kidney fibroblasts) were maintained in Dulbecco's modified Minimum Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum, 50 mU/ml penicillin and 50 ng/ml streptomycin. Low passage (p10) herpes simplex virus type-1 (HSV-1) strain KOS was used throughout this study. Viral stocks were prepared and titrated on monolayers of Vero cells.

4.2.2. HSV-1 infection.

5×10^5 Vero cells were infected with 50 plaque forming units (PFU) of HSV-1, strain KOS per cell in serum-free media, unless otherwise indicated. After 1h at 37°C, the inocula were removed and cells were washed twice with cold phosphate-buffered saline (PBS – 1 mM KH_2PO_4, 154 mM NaCl, 3 mM Na_2HPO_4, pH 7.4). Cells were then incubated in complete medium supplemented with the indicated concentrations of cycloheximide (CHX), phosphonoacetic acid (PAA), or roscovitine (Rosco).

4.2.3. Drugs.

CHX was purchased from Sigma (Oakville, Ontario, Canada), and prepared in serum-free DMEM as a 5 mg/ml stock and stored at 4°C. The stock was diluted to 50 μg/ml in complete medium and added to cells 1h prior to infection. The racemic Rosco mixture was purchased from LC laboratories (Woburn, MA, USA) and was prepared in

DMSO as 100 mM stock, stored at -20°C and used at 400 μM. Phosphonoacetic acid (PAA) was purchased from Sigma (Oakville, Ontario, Canada). PAA was prepared in serum-free DMEM as a 100 mg/ml stock, stored at -20°C, and used at 400 μg/ml in complete media.

4.2.4. Hybridizations.

Vero cells were seeded in 100mm diameter dishes the night prior to infection and infected with 20 PFU of HSV-1. Infected cells were treated with complete medium supplemented or not with 400 μg/ml of PAA. Cells were harvested 6 or 12h later, resuspended in STE buffer (1 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA) and digested with 200 μg/ml proteinase K in 0.5% SDS for 5h at 55°C. DNA was extracted once in phenol:chloroform:isoamyl alcohol (25:24:1) and once in chloroform:isoamyl alcohol (24:1). DNA was then precipitated in the presence of 2.5 volumes of 100% ethanol at -20°C for 5h, and resuspended in TE pH 7.5 (10 mM Tris-Cl, 1mM EDTA). Samples were run on a 0.6% agarose gel and blotted onto nylon membranes following standard protocols. Membranes were pre-hybridized with rapid hybrid buffer (Amersham Biosciences, NJ, USA) at 80°C, then hybridized with ^{32}P-dCTP labeled probe (a mixture of ICP0, ICP4, ICP8, and gC specific probes Diwan et al., 2004), in rapid hybrid buffer for 2h at 80°C. Membranes were washed at room temperature twice for 15 min each in 300 mM NaCl, 30 mM sodium citrate (2 × SSC), 0.1% SDS. If needed, membranes were then washed once for 15 min in 75 mM NaCl, 7.5 mM sodium citrate (0.5 × SSC), 0.5% SDS at 80°C. Membranes were exposed to Kodak PhosphorImager screens and scanned in a Molecular Imager FX (BIO-RAD, Mississauga, Canada).

Signal intensity was quantitated using Quantity One software (BIO-RAD, Mississauga, Canada) and expressed as arbitrary units.

4.2.5. Immunoprecipitation assays.

Vero cells were seeded in 35mm diameter dishes the night prior to infection and mock infected or infected with 50 PFU of HSV-1 per cell in complete DMEM or methionine-free DMEM (Gibco) supplemented with 50 μCi/mL ^{35}S-methionine, as indicated. Cells in two infected dishes treated with 50 μCi/mL ^{35}S-methionine were harvested at 5 hpi in RIPA buffer (150mM NaCl, 50mM Tris pH 7.5, 0.1% SDS, 1% NP-40, and 0.5% deoxycholic acid) supplemented with phosphatase and protease inhibitors. Cells in the other dishes were then treated for 2h with 0 or 100 μM Rosco, together with 50 μCi/mL ^{35}S-methionine in methionine-free DMEM (Gibco), and then harvested in RIPA buffer supplemented with phosphatase and protease inhibitors. Cell lysates were counted in a liquid scintillation counter. Approximately 100 µg of cell lysates containing equal levels of radioactivity were precleared for 1h at 4°C with anti-albumin antibody (a generous gift from Dr. Richard Lehner, University of Alberta, Edmonton, Alberta, Canada) diluted 1:1000 in RIPA buffer. Afterwards, 40 μL/mL protein A conjugated agarose beads slurry (Amersham Biosciences) was added and the extracts were further incubated at 4°C for 1h. Proteins binding to the irrelevant antibody were centrifuged and the supernatants were transferred to fresh tubes. Supernatants were then incubated at 4°C for 1h with monoclonal anti-ICP4 antibody (clone 1101 897 – Rambaugh-Goodwin Institute for Cancer Research, Inc., Plantation, FL, USA) diluted 1:1000 in RIPA buffer. Afterwards, supernatants were further incubated overnight at 4°C

with 30 μL/mL protein A conjugated agarose beads slurry (Amersham Biosciences). Proteins binding specifically to the ICP4 antibody were then centrifuged. Pellets were washed once with RIPA buffer and once with PBS prior to resuspending in 40 μL SDS-PAGE gel loading buffer (200mM Tris-Cl pH 6.8, 8% SDS, 0.2% bromophenol blue, 20% glycerol, and 400mM DTT). Samples were run on a 7.5% SDS-PAGE gel, dried and exposed to phosphoimager.

4.3. RESULTS

4.3.1. HSV-1 DNA replication was efficiently inhibited by PAA in the experiments described in Chapter 3.

To evaluate whether HSV-1 DNA replication was efficiently inhibited by 400 μg/ml PAA in the experiments used in Chapter 3, infected cells were incubated in complete media supplemented or not with 400 μg/ml PAA. Cells were harvested 6 or 12h later and HSV-1 DNA levels were evaluated by Southern blot. As expected, HSV-1 DNA replication was inhibited by 400 μg/ml PAA **(Figure 4.1A and B).**

To test whether HSV-1 DNA replication was efficiently inhibited by 400 μg/ml PAA after the removal of CHX used in Chapter 3, I overexpressed IE proteins using the CHX release protocol. Cells were then transferred to complete media supplemented with 0 or 400 μg/ml PAA at 6 hpi. Infected cells were harvested 6 or 12h later and HSV-1 DNA levels were evaluated by Southern blot.

As expected, HSV-1 DNA replication was inhibited by 400 μg/ml PAA in the experiments used in chapter 3 **(Figure 4.2A and B).**

4.3.2. Ongoing HSV-1 DNA replication was efficiently inhibited by PAA in the experiments used in Chapter 3.

To evaluate whether ongoing HSV-1 DNA replication was efficiently inhibited by 400 μg/ml PAA in the experiments used in Chapter 3, cells were incubated at 5 hpi with complete media supplemented with 0 or 400 μg/ml PAA. Infected cells were then harvested 6 or 12h later and HSV-1 DNA levels were evaluated by Southern blot.

As expected, ongoing HSV-1 DNA replication was efficiently inhibited by 400 μg/ml PAA in the experiments used in Chapter 3 **(Figure 4.3A and B).**

4.3.3. Inhibition of ICP4 phosphorylation by roscovitine does not affect ICP4 binding to a small set of binding proteins.

I next tested whether ICP4 phosphorylation affects its interactions with other proteins. Mock-infected or HSV-1 infected cells were incubated with drug free media for 5h in the presence of ^{35}S-methionine, to allow expression and labeling of IE and E proteins (and formation of RCs) prior to drug treatment. Infected cells were then further incubated for 2h with complete media supplemented with 0 or 100 μM Rosco. Cells were then harvested and proteins were extracted in RIPA buffer containing protease and phosphotase inhibitors. Equal levels of radioactivity, containing approximately 100 μg of each sample, were then immunoprecipitated with ICP4 antibodies. Immunoprecipitated proteins were resolved on 7.5% SDS-PAGE gels, dried, and evaluated by phosphoimager **(Figure 4.4)**.

Two background bands were observed in mock-infected cells, most likely due to cross-reactivity of the ICP4 antibody with a cellular protein. As expected, several

proteins co-immunoprecipitated with ICP4 in extracts from cells infected in the absence of any drug. Surprinsingly, Rosco did not affect co-immunoprecipitation of any of these proteins with ICP4.

4.4. DISCUSSION

Inhibition of HSV-1 DNA replication by 400 μg/ml of PAA prevented ICP4 localization into RCs, even when ICP4 was overexpressed or when RCs were preformed prior to addition of the drug **(Chapter 3).** This concentration of PAA also prevented accumulation of ICP8 into RCs, and thus the formation of these compartments **(Chapter 3).** In the experiments described in this chapter, I have confirmed that HSV-1 DNA replication was inhibited in the experimental conditions using 400 μg/mL of PAA, as expected **(Figures 4.1, 4.2, and 4.3).**

Since ICP4 binding to its cognate sequences was not sufficient for its recruitment to RCs, I further tested whether binding to other proteins was sufficient. I found that inhibition of ICP4 phosphorylation by Rosco did not affect the interactions of ICP4 with a set of binding partners **(Figure 4.4).** The top band in the gel (denoted by the first arrowhead) is most likely ICP4 itself. The second band from the top (denoted by the second arrowhead – approximately 165 kDa) is most likely ICP0. Although the theoretical mass of ICP0 is approximately 110 kDa, ICP0 migrates at a variety of molecular weights under different gel conditions. Furthermore, Rosco was also shown to alter the electrophoretic mobility of a portion of ICP0 molecules (Davido et al., 2002). Moreover, ICP4 and ICP0 are known to physically interact with each other (Yao and Schaffer, 1994). The bottom two arrowheads (approximately 121 kDa) are most

probably ICP8 (approximately 128 kDa). ICP8 has two conformational isomers (Knipe et al., 1982), which could explain the two bands in the gel. It has been previously demonstrated that ICP4 and ICP8 co-immunoprecipitate (Tang et al., 2003). Therefore, the ICP4 interactions with these specific binding partners are not affected by Rosco and therefore may not be sufficient for its recruitment to RCs.

4.5. REFERENCES

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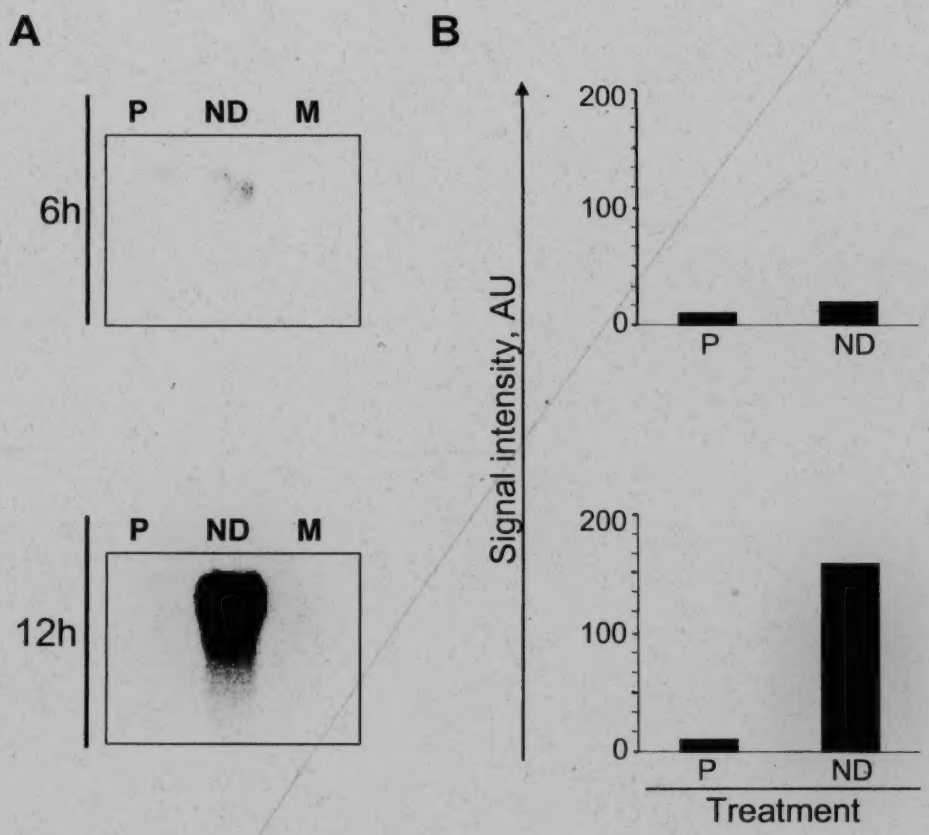

Figure 4.1. HSV-1 DNA replication was efficiently inhibited by PAA in the experimental conditions presented in figure 3.2.

Two Southern blots from a representative experiment showing HSV-1 DNA levels at 6 and 12 hours post-drug treatment **(A)** and two bar graphs representing the quantitation of 2 independent experiments **(B)** are presented above. Vero cells were infected with 10 PFU of HSV-1 per cell. After 1h, cells were treated with no drug **(ND)** or 400 μg/ml PAA **(P)**. Viral DNA was harvested at 7 and 13 hpi, resolved by agarose gel electrophoresis, blotted, and hybridized with HSV-1 specific probes **(A).** Two bar graphs presenting quantitation of bands of one Southern blot expressed as arbitrary units (AU), plotted against drug treatment **(B)**. Results representative of two independent experiments are shown.

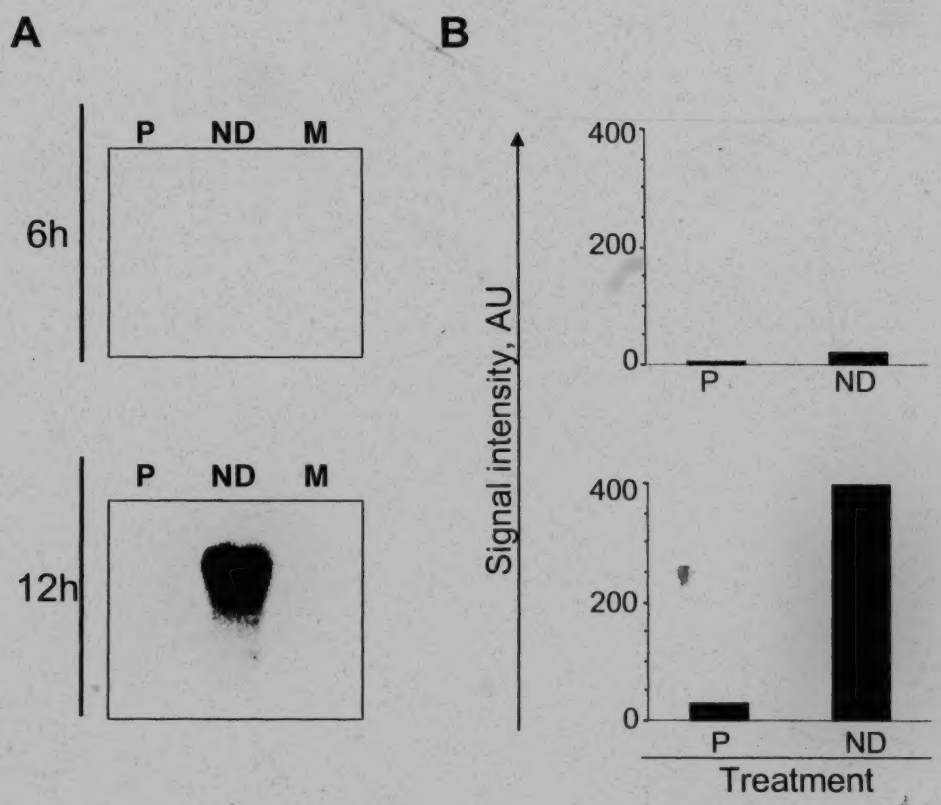

Figure 4.2. HSV-1 DNA replication was efficiently inhibited by PAA in the experimental conditions used in figure 3.4.

Two Southern blots from a representative experiment showing HSV-1 DNA levels at 6 and 12 hours post-drug treatment **(A)** and two bar graphs representing the quantitation of two independent experiments **(B)** are presented above. Vero cells were infected with 10 PFU of HSV-1 per cell in CHX containing media. At 6 hpi, CHX containing media was removed and cells were treated with no drug **(ND)** or 400 μg/ml PAA **(P)**. Viral DNA was harvested at 12 and 24 hpi, resolved by agarose gel electrophoresis, blotted, and hybridized with HSV-1 specific probes **(A).** Two bar graphs, representative quantitation from bands of one Southern blot, presenting signal intensity, expressed as arbitrary units (AU), plotted against drug treatment **(B).** Results representative of two independent experiments are shown.

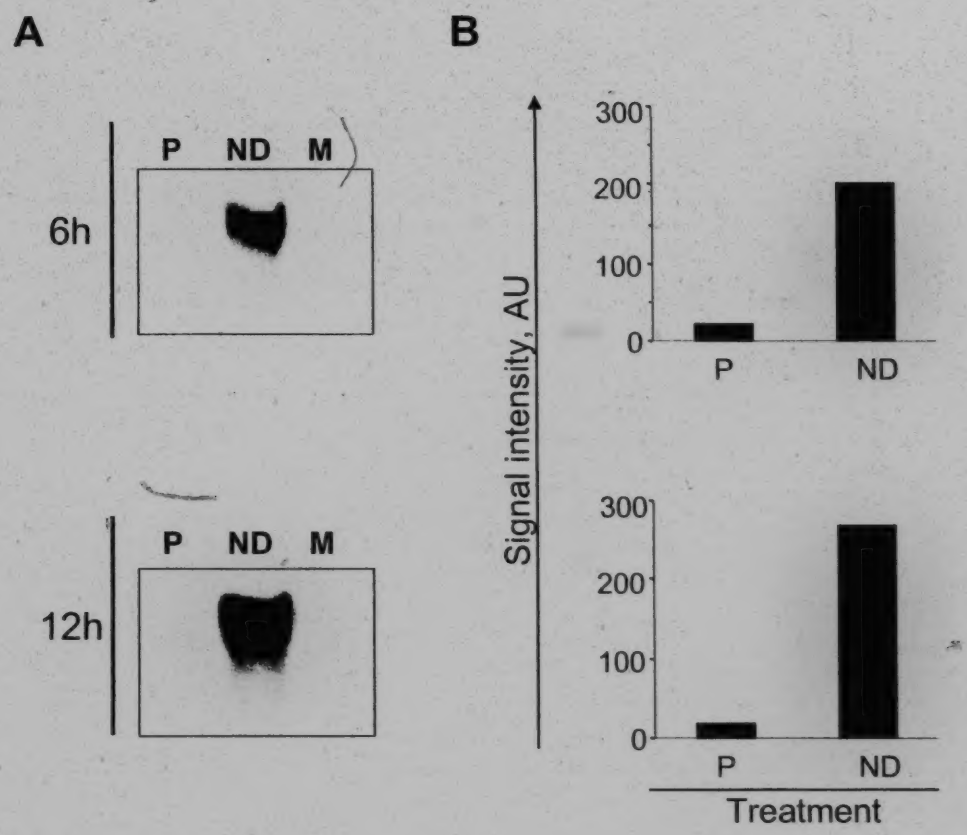

Figure 4.3. Ongoing HSV-1 DNA replication was efficiently inhibited by PAA in the experimental conditions presented in figure 3.9.

Two Southern blots from a representative experiment showing HSV-1 DNA levels at 6 and 12 hours post-drug treatment **(A)** and two bar graphs representing the quantitation of two independent experiments **(B)** are presented above. Vero cells were infected with 10 PFU of HSV-1 per cell. At 5 hpi, cells were treated with no drug **(ND)**, or with 400 μg/ml PAA **(P_H)**. Viral DNA was harvested at 11 and 17 hpi, resolved by agarose gel electrophoresis, blotted, and hybridized with HSV-1 specific probes **(A)**. Two bar graphs representing quantitation from bands of one Southern blot, presenting signal intensity, expressed as arbitrary units (AU), plotted against drug treatment **(B)**. Results representative of two independent experiments are shown.

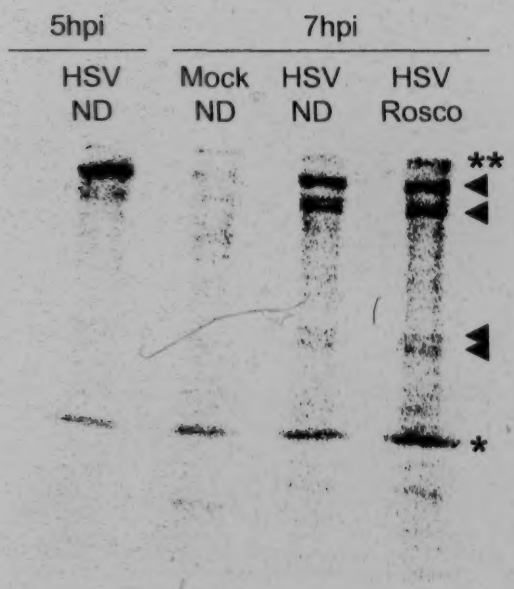

Figure 4.4. Roscovitine does not affect ICP4 binding to two other proteins.

One autoradiograph presenting proteins co-immunoprecipitated with ICP4. Vero cells were infected with 50 PFU of HSV-1 per cell in the presence of ^{35}S-methionine. Cells were treated with no drug (ND) or 100 μM Rosco (Rosco) for 2h starting at 5hpi. Proteins were harvested in the presence of phosphatase and protease inhibitors. Equal radioactivity levels were immunoprecipitated with ICP4 antibody and resolved on a 7.5% SDS-PAGE gel, dried, and evaluated by phosphoimager.

* proteins present in all lanes

◀ proteins present in infected cells only

CHAPTER 5: DISCUSSION

Although HSV-1 is a well studied virus, certain aspects of its replication still remain incompletely understood. One such aspect is the mechanisms of recruitment of ICP4 into replication compartments (RCs). Therefore, I directed my studies at analyzing the importance of phosphorylation in such recruitment.

Replication in defined nuclear domains is not unique to HSV-1. For example, adenoviruses also form homologous nuclear structures. During adenovirus infection, one nuclear subclass of the viral DNA binding protein (DBP) localizes to small nuclear domains within 12 hpi. These domains then increase in number and size as infection proceeds, and are the sites of adenovirus DNA replication. The formation of "DBP-containing domains" is prevented when adenovirus DNA replication is blocked by hydroxyurea. Therefore, this subclass of DBP participates in ongoing viral DNA replication (Voelkerding and Klessig, 1986). Analogously, HSV-1 RCs are also involved in HSV-1 DNA replication, and they are disrupted in the presence of PAA (a DNA synthesis inhibitor). PAA further inhibits the localization of the HSV-1 DNA-binding protein ICP8 to RCs **(Figure 3.8)**.

The adenovirus replication compartments may also be the sites where adenovirus gene expression occurs, in that they are partially disrupted in the presence of actinomycin D (a transcription inhibitor) (Voelkerding and Klessig, 1986). HSV-1 RCs are also the sites of HSV-1 gene expression (Rice, 1994; Leopardi et al., 1997; Phelan et al., 1997). Like adenovirus replication compartments, HSV-1 RCs were disrupted by inhibition of HSV-1 transcription. The pharmacological cyclin-dependent kinase (CDK) inhibitor (PCI), roscovitine (Rosco) inhibits initiation of transcription of immediate-early (IE) and

early (E) HSV-1 genes (Diwan et al., 2004; Lacasse et al., 2005). Consequently, Rosco, and other PCIs such as Flavo, prevent expression of ICP4 **(Figure 2.2)** without significant cytotoxicity **(Figure 2.1 and Table 2.1)**. Rosco and Flavo also prevented the accumulation of ICP4 into RCs **(Figure 2.3)**. This suggests that two different PCIs, which inhibit different subsets of CDKs, inhibit the localization of ICP4 to RCs.

Rosco also affects subnuclear localization of proteins of varicella-zoster virus (VZV). It was shown that Rosco prevented normal localization of VZV ICP4 homologue, IE62 (Taylor et al., 2004) and VZV ICP22 homologue, IE63 (Habran et al., 2005). Rosco also inhibits gene expression of IE62 (Taylor et al., 2004). My finding that Rosco prevented accumulation of ICP4 into RCs is fully consistent with the results reported by Taylor *et al.* (2004) **(Figures 2.3, 3.1, and 3.2).** But Rosco also inhibited expression of VZV IE62. Therefore, the abnormal localization of VZV IE62 may have been exclusively secondary to its reduced levels of expression. Even though ICP4 expression levels also decreased in the presence of Rosco (and Flavo) **(Figure 2.2)**, I have shown that over-expression of ICP4 did not overcome the effect of Rosco on ICP4 accumulation into RCs **(Figure 3.4).** Therefore, the effect of Rosco on ICP4 recruitment into RCs was not exclusively a consequence of its effects on protein level **(Figure 3.3).** By analogy, VZV IE62 may also require Rosco-sensitive kinases for its proper localization into replication compartments, beyond the effects of Rosco on its expression levels.

Like that of HSV-1 ICP4, phosphorylation of VZV IE63 was also inhibited by Rosco, and by another "CDK1 specific" inhibitor (Habran et al., 2005). This

phosphorylation was shown to be important for IE63 repression of the VZV DNA polymerase promoter (Habran et al., 2005).

Since ICP4 phosphorylation had been reported to be inhibited by Rosco at late times post infection (Advani et al., 2001), I used Rosco to test whether ICP4 phosphorylation was required for its recruitment into RCs. I have shown in this thesis that Rosco inhibits ICP4 phosphorylation at the times of infection when ICP4 is recruited to RCs, and when it activates or represses transcription.

Rosco efficiently inhibits CDK1, 2, 5, 7, and perhaps 9 (Meijer et al., 1997; Wang et al., 2001; Schang et al., 2002a). Of these CDKs, CDK5 is inactive in cycling cells, where PCIs efficiently inhibit HSV-1 replication and CDK1 is not required for the expression of the majority of HSV-1 genes (Advani et al., 2000). Schang *et al.*, (2002b) showed that CDK2, cyclin A, and cyclin E are expressed in explanted neurons that supported HSV-1 replication. In contrast, HSV-1 replicated in neurons expressing no CDK1 or CDK7. These data suggest that CDK2 may be one of the kinases involved in HSV-1 replication, at least in neurons.

To evaluate whether the differential recruitment of phosphorylated ICP4 was a result of novel protein-protein interactions I tested the ability of Rosco to inhibit ICP4 phosphorylation after CHX removal **(Figure 3.5)** and after formation of RCs **(Figure 3.8)**. I suggest that the phosphoproteins observed in the experiments presented in figure 3.5 were all IE proteins. The molecular weights of the observed phosphoproteins match those of the IE proteins described by many others on similar SDS-PAGE gels (Honess and Roizman, 1973; Honess and Roizman, 1974) and these experiments were performed using a "CHX release experimental design". CHX only allows accumulation of IE

transcripts, and Rosco inhibits IE and E gene transcription (Diwan et al., 2004). Therefore, E or L proteins were not likely expressed in the experiment presented in figure 3.5. I thus further propose that Rosco also inhibited phosphorylation of all other four IE proteins, although to different extents **(Figure 3.5)**. The significance of the inhibition of phosphorylation of all IE proteins by Rosco was not pursued, however, since it lies outside the scope of this thesis.

It has been hypothesized that CDK1 phosphorylates ICP4 into the most hyperphosphorylated form, phosphorylation that was inhibited by Rosco (Advani et al., 2001). However, this Rosco-sensitive hyperphosphorylated ICP4 was only detected at 10 hpi or later. I have shown now that Rosco also inhibits phosphorylation of ICP4 as early as 5 to 7 hpi, and as early as 4 hours after the removal of CHX **(Figures 3.5 and 3.8)**. I have not studied earlier times, but it appears likely that Rosco may have inhibited ICP4 phosphorylation even before these times. Phosphorylation of ICP4 in the absence of drug was also decreased at 7 hpi in comparison to 5 hpi **(Figure 3.8)**, which is expected since phosphorylation of ICP4 is dynamic.

Advani *et al.* (2001) suggested that CDK1 was the major ICP4 kinase based on *in vitro* kinase assays, which showed a decrease in CDK1 activity in the presence of increasing concentrations of Rosco. In those experiments, CDK2 activity did not significantly decrease until the addition of 100 μM of Rosco. However, Rosco is known to inhibit CDK2 activity with an IC_{50} of 0.7 μM (Meijer et al., 1997) and thus it would be expected that CDK2 activity would have been partially inhibited at 10 μM (the other concentration of Rosco tested). CDK2 activity may well be inhibited during HSV-1 infection, but such inhibition is at most only partial and occurs at late times post-infection

(8 to 12 hours post-infection) (Ehmann et al., 2000; Advani et al., 2001; Ehmann et al., 2001). In contrast, PCIs inhibited ICP4 accumulation into RCs as early as 3 hpi **(Figure 3.2)**. Therefore, the levels of kinase activities at late times during infection provide little information with respect to which Rosco-sensitive kinases may be required for ICP4 the recruitment of ICP4 into RCs.

Phosphorylation of ICP4 was inhibited by approximately 66% in the presence of Rosco **(Figure 3.8)**. This inhibition paralleled the extent of inhibition of ICP4 recruitment into RCs by Rosco, in that further recruitment of ICP4 into RCs was also inhibited by approximately 60% in the presence of Rosco **(Figure 3.9B)**. Thus, my current hypothesis is that ICP4 phosphorylation is most likely required for its recruitment into RCs.

Although Rosco efficiently inhibited ICP4 phosphorylation, phosphorylation was not reduced to background levels when Rosco was added at 5 hpi **(Figure 3.8)**. In contrast, Rosco efficiently inhibited ICP4 phosphorylation when added immediately after removal of CHX **(Figure 3.5)**. In the former experiment, phosphorylated ICP4 is present prior to the addition of Rosco. Although I can not observe this phosphorylated ICP4 since ^{32}P was only added in the presence of Rosco, I cannot exclude the possibility that these phosphorylated ICP4 proteins could indirectly affect inhibition of ICP4 phosphorylation when in the presence of Rosco. These results may also suggest that Rosco-insensitive pathways are also involved in ICP4 phosphorylation. cAMP-dependent protein kinase A (PKA) has been suggested to phosphorylate ICP4 within the serine-rich region (Mullen et al., 1995; Xia et al., 1996a; Xia et al., 1996b) and this domain also contains consensus sites for protein kinase C (PKC) and casein kinase II

(CKII) (Xia et al., 1996a; Xia et al., 1996b). None of these other kinases has been tested with regard to their involvement in the recruitment of ICP4 into RCs. Furthermore, an ICP4 phosphorylation cascade may exist in which multiple protein kinases, including perhaps Rosco-sensitive kinases, may be involved.

It has been demonstrated that phosphorylation of ICP4 is not required for binding of ICP4 to its high affinity binding sites in the IE promoters (Papavassiliou et al., 1991). Although the ICP4 DNA binding domain is essential for activation of transcription (Paterson and Everett, 1988; Shepard et al., 1989), extensive research has failed to reveal any specific sequence common to all promoters activated by ICP4 that is specifically recognized by ICP4 (Everett, 1987; Wagner et al., 1995). ICP4 binding to DNA further depends on the length and on the proximity of the binding site to the fragment end (Michael and Roizman, 1989), which was considered in the design of the oligonucleotides used in the gel mobility shift assays **(Figure 3.6)**. The oligonucleotide probe contained the ICP4 binding site fourteen base pairs away from the 3'-terminal end and nine base pairs away from the 5'-terminal end.

The high affinity consensus sequence of the ICP4 binding sites in HSV-1 IE promoters is ATCGTC (Faber and Wilcox, 1986a). This sequence is conserved among several herpesviruses, as is its recognition by the respective ICP4 homologues, VZV IE62 and pseudorabies IE180 (Wu and Wilcox, 1991). Such high degree of conservation suggests the importance of the high affinity sequence specific DNA binding of ICP4. It is thus perhaps surprising that the importance of ICP4 DNA binding for its recruitment into RCs (which contain large numbers of HSV-1 genomes) had not been directly analyzed before. However, Everett et *al.* have studied the colocalization of ICP4 with

HSV-1 genomes (Everett et al., 2004). I have shown now that ICP4 binding to its cognate sites is not sufficient for recruitment to RCs, in that ICP4 bound to its cognate sites even in the presence of Rosco, which prevented its recruitment into RCs **(Figure 3.6)**. These results were perhaps expected, since both phosphorylated ICP4 and ICP4 dephosphorylated *in vitro* by phosphatases had previously been shown to bind ICP4 binding sites (Michael et al., 1988; Papavassiliou et al., 1991). Furthermore, deletion of residues 143-210 (Shepard et al., 1989) or residues 162-229 (Paterson et al., 1990), which overlap the serine-rich region, does not affect binding to high affinity binding sites. Furthermore, deletion of residues 143-210 inhibits activation of transcription of E genes (Shepard et al., 1989).

Rosco prevents initiation of transcription of IE genes **(Figure 2.4)**, inhibition which is not a consequence of inhibition of Oct-1/HCF/VP16 binding to their cognate sequences (Jordan et al., 1999; Diwan et al., 2004). In two different systems, thus, Rosco inhibited transactivation by DNA binding proteins without affecting their binding to their cognate sites.

Since ICP4 binding to its cognate sites does not appear to be sufficient for its recruitment to RCs, I therefore tested next whether protein-protein interactions were sufficient. I have shown that Rosco does not disrupt binding of ICP4 to a limited set of binding partners, likely those proteins binding with the highest affinity **(Figure 4.4)**. I suggest that these binding partners could be ICP0 or ICP8 which have been shown previously to co-immunoprecipitate with ICP4. However, I cannot be certain of the identity of these proteins. In order to identify these bands, western blot or mass spectrometry analyses should be performed. ICP4 is also known to form homodimers

and perhaps even oligomers (Metzler and Wilcox, 1985; Faber and Wilcox, 1986b; Kattar-Cooley and Wilcox, 1989; Shepard et al., 1990). To analyze whether ICP4 co-immunoprecipitated with another ICP4 molecule I would have to transfect two plasmids containing ICP4 tagged with two different tags and perform a co-immunoprecipitation using an antibody against one of these tags and a western blot using an antibody against the other. Inhibition of ICP4 phosphorylation prevented its recruitment into RCs but did not prevent binding to two other proteins. Therefore, binding to these proteins is not sufficient for the recruitment of ICP4 into RCs.

ICP8 is one of the first proteins that accumulate into RCs. Thus, the ability of Rosco to prevent ICP4 accumulation to RCs could have resulted from its inhibition of formation of RCs. However, Rosco had no major effects on further accumulation of ICP8 into RCs when the drug was added after RCs had formed **(Figure 3.9A)**, conditions under which Rosco did inhibit further accumulation of ICP4 **(Figure 3.9B)**. Therefore, inhibition of ICP4 recruitment to RCs in these experiments by Rosco was not exclusively a consequence of the effects of Rosco on RC formation.

It has been previously shown that inhibition of HSV-1 DNA replication by PAA prevents the localization of ICP8, to RCs and thus the formation of these compartments (de Bruyn Kops et al., 1998), and Rosco inhibits HSV-1 DNA replication (Schang et al., 2000). Therefore, the effects of Rosco on recruitment of ICP4 into RCs could have been exclusively secondary to its ability to inhibit HSV-1 DNA replication. High concentrations of PAA inhibited HSV-1 DNA replication better than Rosco in all experimental conditions used and also inhibited ICP4 recruitment better **(Figures 4.1 to 4.3)**. However, high concentrations of PAA, unlike Rosco, also prevented the formation

of RCs thus preventing ICP4 accumulation into RCs **(Figure 3.9)**. When low concentrations of PAA were added after infection or after CHX treatment, Rosco inhibited HSV-1 DNA replication better than PAA in these experiments. These results suggest that the effects of Rosco and PAA on ICP4 recruitment in these conditions could have been exclusively secondary to the effects on DNA replication. However, low concentrations of PAA added after formation of RCs resulted in similar levels of inhibition of HSV-1 DNA replication as those achieved by Rosco **(Figure 3.12A and B)**, but did not inhibit equally well the accumulation of ICP4 into RCs **(Figure 3.9B)**. The inhibition of ICP4 recruitment to RCs by Rosco observed in these latter experiments did, thus, not result exclusively from the effects of Rosco on of HSV-1 DNA replication.

In this thesis I have thus demonstrated that ICP4 phosphorylation is most likely required for its recruitment into RCs. ICP4 phosphorylation was inhibited by Rosco, further suggesting that it is mediated by Rosco-sensitive kinases. I have not attempted to evaluate whether the participation of such kinases was direct or indirect. I hence propose that the phosphorylation of ICP4 involves a cascade involving Rosco-sensitive kinases. These Rosco-sensitive kinases could potentially phosphorylate ICP4 directly, or more likely, phosphorylate other proteins which could then in turn mediate the phosphorylation of ICP4. I have further shown that ICP4 binding to its cognate DNA sites, or to two high-affinity binding proteins, is not sufficient for its recruitment to RCs.

FUTURE DIRECTIONS

ICP4 is a phosphoprotein with many potential phosphorylation sites. These sites include several residues in the N-terminal serine-rich region, which contains consensus

sites for PKA, PKC, and CKII. However, it is yet unknown which kinases are involved in the ICP4 phosphorylations sufficient for its recruitment into RCs. The identification of these kinases would allow us to further evaluate the mechanism whereby ICP4 is recruited into RCs. The kinases involved could be identified using in gel kinase assays, biochemical purification, or siRNA screening. After such identification, we could analyze the ability of the identified kinases to phosphorylate ICP4 in kinase assays *in vitro*.

Tandem mass spectrometry could be used to identify the ICP4 sites that are phosphorylated *in vivo*. Similar approaches were successfully used for another IE protein, ICP0 (Davido et al., 2005). The phosphorylation sites could then perhaps be confirmed via a newly described cross-linking kinase assay which allows covalent cross-linking between the substrate and the upstream kinase *in vivo* (Maly et al., 2004).

The proteins co-immunoprecipitated with ICP4 in figure 4.4 could be identified to analyze which proteins are involved in the phosphorylation cascade of ICP4 and thus in its recruitment into RCs. The identification of ICP4 binding proteins could provide further information on a possible ICP4 phosphorylation cascade which is required for its recruitment into RCs. However, it is also entirely possible that the proteins identified are not involved in ICP4 recruitment into RCs. This appears especially likely since I did not observe differences between ICP4 binding to two proteins in the presence of Rosco **(Figure 4.4)**.

A biochemical identification of the kinases involved in ICP4 phosphorylation could be attempted. This method would identify protein fractions (nuclear or cytoplasmic) that phosphorylate ICP4. To accomplish this, cell lysates would be

fractionated into nuclear and cytoplasmic fractions. ICP4, $^{32}P_i$, and proper kinase buffer would be added to an aliquot of each fraction. These reactions would then be run on a SDS-PAGE to identify which fraction phosphorylates ICP4. The phosphorylating fractions would subsequently be sub-fractionated, and each sub-fraction would be similarly analyzed. The process would be repeated until the most purified kinase preparation is identified. This fraction would then be resolved in SDS-PAGE, and all proteins in this fraction can be excised and identified by mass spectrometry.

Another method suitable for the identification of the kinases involved in ICP4 phosphorylation is in gel kinase assays. Cellular protein preparations are resolved on SDS gels containing cross-linked ICP4. If multi-subunit kinases are considered as likely candidates, non denaturing gels could be used instead. After electrophoresis, the proteins in the gel are renatured and incubated with ^{32}P-ATP and appropriate buffer, thus allowing the candidate kinases to phosphorylate ICP4. The phosphorylated bands can be detected by autoradiography and the potential kinases identified by molecular weight and western blot (Wooten, 2002).

GST-pulldowns are yet another method suitable for the identification of the proteins involved in ICP4 phosphorylation. ICP4-GST fusions would be constructed and incubated with cell lysates from HSV-1- or mock-infected cells. This method would reveal potential ICP4 binding partners. However, this method is not likely to identify the protein kinases that phosphorylate ICP4. Binding of protein kinases to their substrates is transient and thus it is often difficult to pull them down with their substrates.

siRNA against the putative kinases could then be used to test their roles in ICP4 recruitment *in vivo*. As potential drawbacks, siRNAs may result in insufficient depletion, or in secondary effects.

The proteins co-immunoprecipitated with ICP4 **(Figure 4.4)** could be identified. Even though I have suggested that these proteins are likely ICP0 or ICP8, I cannot confirm that these are indeed the proteins observed. Mass spectrometry or western blots could be used to confirm their identities. Mass spectrometry may also identify previously unidentified binding partners. This binding partner could be further confirmed by western blot. However, the proteins co-immunoprecipitating with ICP4 may or may not be involved in ICP4 phosphorylation.

Microcapillary high pressure liquid chromatography tandem mass spectrometry (μLC-MS/MS) can be used to identify the individual phosphorylation sites on ICP4. Purified ICP4 would be digested in-gel with trypsin and chymotrypsin. The resulting digests would then be pooled and analyzed by μLC-MS/MS. MS/MS spectra would identify the phosphorylated peptides, which can then be sequenced. The specific site of phosphorylation on these peptides can then be identified. Since covalent attachment of a phosphate group increases mass by 80Da, this will change the charge-to-mass ratio, change that will be detected in the peptide spectra. This method has been used successfully by Davido *et al.* (2005) to determine the major phosphorylation regions in another HSV-1 IE protein, ICP0.

Chemical cross-linking of kinases has now been improved and been performed with success (Maly et al., 2004). Kinase-substrate cross-linking involves the mutation of the phosphorylated serine or threonine in the substrate to a cysteine. This method utilizes

a cross-linker that links the so mutated substrate to the catalytic lysine residue of the kinase, therefore, making the kinase-substrate interaction covalent. The cross-linker developed by the authors allows general binding of any kinase. The cross-linker was designed to interact with the conserved ATP-binding site and target the non-variant catalytic lysine residue in the kinase. The kinase-substrate complex could then be identified by immunoprecipitation of ICP4, trypsinization and identification of the cross-linked peptides by mass spectrometry. As a caveat, this new method has been reported only once (Maly et al., 2004).

I have already shown that phosphorylation of ICP4 is most likely required for its recruitment into RCs. As a consequence of my studies, the mechanisms for such phosphorylation and its regulation of ICP4 recruitment now deserve further studies. The evaluation of the mechanism whereby ICP4 is phosphorylated would further characterize of the mechanisms whereby ICP4 is recruited into RCs. These studies on ICP4 may also clarify the mechanisms whereby other HSV-1 proteins, or ICP4 homologues in other herpesviruses, are recruited into RCs or equivalent domains.

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